Environmental Research 169 (2019) 7–25

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Living in a multi-stressors environment: An integrated biomarker approach to assess the ecotoxicological response of meagre (*Argyrosomus regius*) to venlafaxine, warming and acidification

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ARTICLE INFO

Keywords:
Venlafaxine
Fish
Warming
Acidification
Integrated multi-biomarker response

ABSTRACT

Pharmaceuticals, such as the antidepressant venlafaxine (VFX), have been frequently detected in coastal waters and marine biota, and there is a growing body of evidence that these pollutants can be toxic to non-target marine biota, even at low concentrations. Alongside, climate change effects (e.g. warming and acidification) can also affect marine species' physiological fitness and, consequently, compromising their ability to cope with the presence of pollutants. Yet, information regarding interactive effects between pollutants and climate change-related stressors is still scarce. Within this context, the present study aims to assess the differential ecotoxicological responses (antioxidant activity, heat shock response, protein degradation, endocrine disruption and neurotoxicity) of juvenile fish (*Argyrosomus regius*) tissues (muscle, gills, liver and brain) exposed to VFX (via water or feed), as well as to the interactive effects of warming ($\Delta T\,^{\circ}C = +5\,^{\circ}C$) and acidification ($\Delta pCO_2 \sim +1000$ µatm, equivalent to $\Delta pH = -0.4$ units), using an integrated multi-biomarker response (IBR) approach.

Overall, results showed that VFX toxicity was strongly influenced by the uptake pathway, as well as by warming and acidification. More significant changes (e.g. increases surpassing 100% in lipid peroxidation, LPO, heat shock response protein content, HSP70/HSC70, and total ubiquitin content, Ub,) and higher IBR index values were observed when VFX exposure occurred via water (i.e. average IBR = 19, against 17 in VFX-feed treatment). The co-exposure to climate change-related stressors either enhanced (e.g. glutathione S-transferases activity (GST) in fish muscle was further increased by warming) or attenuated the changes elicited by VFX (e.g. vitellogenin, VTG, liver content increased with VFX feed exposure acting alone, but not when co-exposed with acidification). Yet, increased stress severity was observed when the three stressors acted simultaneously, particularly in fish exposed to VFX via water (i.e. average IBR = 21). Hence, the distinct fish tissues responses elicited by the different scenarios emphasized the relevance of performing multi-stressors ecotoxicological studies, as such approach enables a better estimation of the environmental hazards posed by pollutants in a changing ocean and, consequently, the development of strategies to mitigate them.

1. Introduction

The exhaustive exploitation of natural resources, along with the increasing production and release of pollutants into the environment, including the "so called" greenhouse gases (GHG, e.g. CO_2, CH_4, N_2O), have contributed to one of the greatest environmental concerns of our time. According to the latest report of the Intergovernmental Panel on Climate Change (IPCC, 2014), GHG emissions have reached unprecedented levels in the last 50 years, unequivocally causing the warming of the planet, with most of the energy produced in the form of heat being stored in the ocean (only ~1% of the total energy produced within the climate system is stored in the atmosphere). Furthermore,

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https://doi.org/10.1016/j.envres.2018.10.021
Received 6 August 2018; Received in revised form 2 October 2018; Accepted 18 October 2018
Available online 26 October 2018
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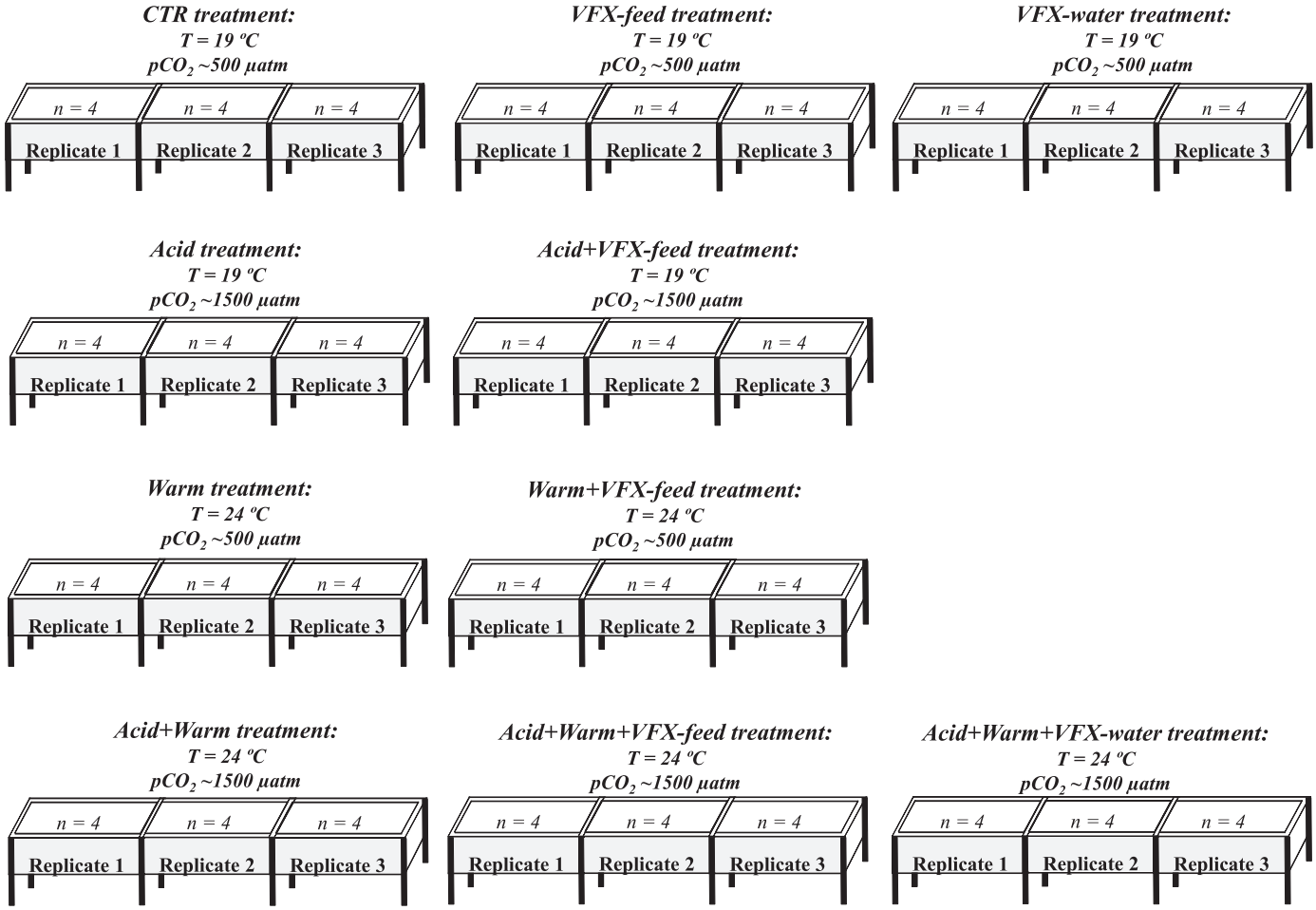

Fig. 1. Experimental setup. Abbreviations: CTR – reference temperature and pH conditions (i.e. T = 19 °C and pH = 8.0 units); VFX-feed – VFX exposure via feed; VFX-water – VFX exposure via water; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 °C).

the increasing release of GHG has also resulted in a higher oceanic uptake of CO_2 (i.e. increased CO_2 partial pressure, pCO_2, which causes the drop of the average seawater pH), therefore, leading to a phenomenon known as "ocean acidification" (IPCC, 2014; McNeil and Sasse, 2016). Thus, even if efforts are made to keep GHG emissions at today's rates and pollution levels stable in a short/medium term, recent projections indicate that, within a 50–100 years' timeframe, seawater temperature and pCO_2 levels will still increase as high as 5 °C and 1000 µatm, respectively (IPCC, 2014; McNeil and Sasse, 2016).

Climate change-related stressors can have negative impacts on marine species, affecting their physiology, metabolism and ecological fitness (e.g. Madeira et al., 2016, Rosa et al., 2014, 2016, 2017), thus, making them less resilient to the co-exposure with other environmental stressors, such as chemical contaminants (Sampaio et al., 2016, 2018; Maulvault et al., 2017, 2018b, 2018c, 2018a; Serra-Compte et al., 2018). On the other hand, changes of the surrounding abiotic conditions (e.g. temperature and pH) can also affect chemical contaminants' physical and chemical properties (i.e. speciation, transport, transfer among compartments), as well as their uptake, elimination and toxicity to marine organisms (e.g. Marques et al., 2010; Maulvault et al., 2016, 2017, 2018a, Sampaio et al., 2018; Serra-Compte et al., 2018). Yet, understanding the potential interactions between climate change-related stressors and pollution is a topic that only recently raised attention within the scientific community and, therefore, further research efforts are urgently required to better forecast the ecotoxicological implications of climate change.

Pharmaceuticals and personal care products (PPCPs) comprise a wide diversity of non-regulated compounds of emerging concern, including human and veterinary pharmaceuticals, cosmetics, preservatives, detergents, among others. These compounds have been frequently detected in coastal waters (concentrations ranging from ng L^{-1} up to mg L^{-1}; e.g. Gaw et al., 2014; Arpin-Pont et al., 2016; Rodriguez-Mozaz et al., 2017), as well as e in marine biota (e.g. Vandermeersch et al., 2015; Rodriguez-Mozaz et al., 2017), promoting several adverse effects in non-target organisms (Best et al., 2014; Bisesi et al., 2014; Bidel et al., 2016; Maulvault et al., 2018b, 2018c). Despite recent evidence suggested that PPCPs' bioavailability and toxicity is strongly mediated by the surrounding abiotic conditions (e.g. Gul et al., 2015; Rowett et al., 2016; Serra-Compte et al., 2018), their ecotoxicological implications to marine organisms under future climate conditions are still ununderstood.

With the aim of better understanding the interactions between PPCPs exposure and abiotic variables, we have recently conducted two studies focused on the widely used psychiatric pharmaceutical venlafaxine (VFX), using juvenile meagre (*Argyrosomus regius*) as model organism (Maulvault et al., 2018a, 2018b). Both of these studies constituted relevant proofs of concept, confirming that the co-exposure to abiotic conditions not only affected VFX's bioaccumulation and elimination mechanisms in fish species (Maulvault et al., 2018a), but also accentuated the behavioural impairments elicited by VFX (Maulvault et al., 2018b). Such findings arose the interest for conducting a third study to assess the potential biochemical alterations at tissue/cell level induced by exposure to VFX, warming and/or acidification. In this context, the present study aimed to investigate the ecotoxicological responses (antioxidant enzymes activities, chaperoning and protein degradation, neurotoxicity and endocrine disruption) of juvenile *A.*

regius tissues (muscle, gills, liver and brain) after 28 days of co-exposure to VFX (via water, i.e. [VFX] ~ 20 μg L^{-1}, and via feed, i.e. [VFX] ~ 160 μg kg^{-1} dry weight, dw), warming (ΔT °C = +5 °C) and acidification (ΔpCO_2 ~ +1000 μatm, equivalent to ΔpH = − 0.4 units), using an integrated multi-biomarker response (IBR) approach.

2. Materials and methods

2.1. Acclimation

A. regius specimens were reared until the juvenile stage at the aquaculture pilot station of the "Portuguese Institute for the Sea and Atmosphere (EPPO-IPMA, Olhão, Portugal)" using routine hatchery conditions, and were subsequently transported to the aquatic facilities of "Laboratório Maritimo da Guia (MARE-FCUL, Cascais, Portugal)". Here, fish with similar morphometry (total length: 6.8 ± 0.6 cm; total weight 2.6 ± 0.8 g; $n = 120$) were randomly and equitably distributed in 30 rectangular shaped incubating glass tanks (10 treatments × 3 replicate tanks; each tank with 50 L of total volume; Fig. 1) within independent recirculation aquaculture systems (RAS). To maintain seawater quality and abiotic parameters at the adequate levels, each tank was equipped with: i) protein skimmer (Reef SkimPro, TMC Iberia, Portugal); ii) UV disinfection (Vecton 300, TMC Iberia, Portugal); iii) biological filtration (model FSBF 1500, TMC Iberia, Portugal); iv) chemical filtration (activated carbon, Fernando Ribeiro Lda, Portugal); v) automatic seawater refrigeration systems (± 0.1 °C; Frimar, Fernando Ribeiro Lda, Portugal) and submerged digital thermostats (200 W, V2Therm, TMCIberia, Portugal) to control seawater temperature; and vi) individual pH probes (GHL, Germany) connected to a computerized pH control system (± 0.1 pH units; Profilux 3.1 N, GHL, Germany) to monitor seawater pH in each tank (measures every 2 s), and adjust to the adequate levels whenever needed, through the injection of either CO_2 (Air Liquide, Portugal; to decrease pH) or CO_2-filtered air (Stella 200 air pumps, Aqua One Pro, Aqua Pacific UK Ltd, United Kingdom; to increase pH) via submerged air stones displayed in each tank. As routine procedures, every day, fish faeces were cleaned and 25% of seawater total volume was renewed in each tank. Ammonia, nitrite and nitrate levels were checked every week using colorimetric tests (Tropic Marin, USA), and kept below detectable levels, with the exception of nitrates (i.e. kept below 2.0 mg L^{-1}). Furthermore, seawater total alkalinity was measured in every tank on a weekly basis, following the protocol described by Sarazin et al. (1999), and the combination of total alkalinity (AT) and pH was used to calculate carbonate system parameters (average values obtained for each treatment can be consulted in Appendix A, Table A1).

Before beginning the trial, fish were acclimated for a period of 30 days, under the following abiotic conditions: i) dissolved oxygen (DO) > 5 mg L^{-1}; ii) temperature (T °C) = 19.0 ± 0.5 °C; iii) pH = 8.00 ± 0.10 units; iv) salinity = 35 ± 1‰; and v) photoperiod = 12 h light:12 h darkness. Temperature, pH, salinity and DO were checked daily using a multi-parameter measuring instrument (Multi 3420 SET G, WTW, Germany). During acclimation, fish from all treatments were fed ~2% of their body weight (BW) with a non-contaminated fish diet, i.e. with CTR feed (VFX concentration in CTR feed < 0.30 ng g^{-1}, i.e. < the limit of detection, LOD, of the methodology used to determine VFX concentrations in feed samples; Gros et al., 2012; Maulvault et al., 2018a). Details regarding feeds preparation and VFX determination were previously presented in Maulvault et al. (2018a, 2018b). Moreover, feeds nutritional composition is available in Appendix A, Table A2. No mortality was observed during the acclimation period (nor during the trial).

2.2. Experimental setup

The experimental setup used to expose fish to VFX (via diet or water), warming and acidification was similar to the ones previously described by Maulvault et al. (2018a, 2018b). Briefly, the following experimental conditions (acting in isolation or combined) were simulated: i) either the absence of VFX contamination (i.e. non-contaminated treatments) or exposure to this pollutant through two different pathways (VFX uptake from water via inhalation, i.e. VFX-water treatments, or VFX uptake from diet via ingestion, i.e. VFX-feed treatments); and ii) either the temperature and pCO_2 conditions normally used in juvenile meagre rearing conditions in Southern Europe (i.e. 19 °C and ~ 500 μatm pCO_2, equivalent to 8.0 pH units) or the projected seawater warming (i.e. Warm treatments; ΔT°C = +5 °C) and acidification (i.e. Acid treatments; ΔpCO_2 ~ +1000 μatm, equivalent to ΔpH = −0.4 units).

To simulate VFX feed exposure, a VFX-enriched diet (with the same nutritional composition as CTR feed; see Appendix A, Table A1) was prepared by top-coating fish CTR feed pellets with a VFX hydrochloride stock solution ($C_{17}H_{27}NO_2$·HCl, > 98%, Sigma-Aldrich; solubilized in deionized water; detailed description of VFX-enriched feed preparation presented in Maulvault et al., 2018a, 2018b). VFX final concentration in VFX-enriched feed was 161.7 ± 17.1 μg kg^{-1} dry weight (dw). To simulate VFX water exposure, a VFX hydrochloride stock solution was also prepared to daily spike each tank, achieving a final VFX concentration of 20.2 ± 3.8 μg L^{-1} in each tank (in a steady state). The criteria followed to select such concentrations, as well as data regarding the stability assessment of VFX concentrations in both feed and seawater throughout the trial are shown in Maulvault et al. (2018a, 2018b).

To simulate seawater warming and acidification, one week before initiating the trial, seawater temperature and pH were gradually adjusted (+1 °C and −0.1 pH unit per day), until reaching 24 °C and ~1500 μatm pCO_2 (equivalent to pH = 7.6 units) in tanks simulating climate change conditions (Fig. 1), according to the projections of the Intergovernmental Panel for Climate Change (scenario RPC8.5; IPCC, 2014), as well as considering the intervals of future CO_2 amplification scenarios described by McNeil and Sasse (2016). Due to experimental limitations, only VFX dietary exposure was selected to investigate all possible interactions between stressors, in a full cross-factorial design (i.e. Warm + VFX-feed, Acid + VFX-feed and Warm + Acid + VFX-feed treatments; the criteria used to prioritize VFX-feed exposure, over VFX-water exposure, was previously described in Maulvault et al., 2018a, 2018b). Nevertheless, the effect of warming and acidification acting together (i.e. the worst-case scenario) was assessed in treatments simulating both VFX exposure routes (i.e. seawater and dietary exposures; Acid + Warm + VFX-water and Acid + Warm + VFX-feed; Fig. 1).

In summary, ten treatments were carried out ($n = 4$ animals per replicate tank, i.e. 12 fish per treatment; the experimental setup is shown in Fig. 1) during 28 days of trial, i.e. 4 non-contaminated treatments in which fish were exposed to the corresponding temperature and pCO_2 conditions while being daily fed (2% BW) with CTR feed (CTR, Acid, Warm and Acid + Warm treatments), 4 treatments simulating VFX dietary exposure, in which fish were exposed to the corresponding temperature and pCO_2 conditions while being daily fed (2% BW) with VFX-enriched feed (VFX-feed, Acid + VFX-feed, Warm + VFX-feed and Acid + Warm + VFX-feed treatments), and 2 treatments simulating VFX water exposure, in which fish were exposed to seawater daily spiked with a VFX stock solution, as well as to the corresponding temperature and pCO_2 conditions, while being daily fed (2% BW) with CTR feed.

2.3. Samples collection

After 28 days of trial, 6 fish were randomly collected from each treatment (i.e. 2 fish collected from each of the 3 replicate tanks that composed each treatment), euthanized by immersion in an overdosed MS222 solution (2000 mg L^{-1}; Sigma-Aldrich, USA) buffered with sodium bicarbonate (1 g of $NaHCO_3$ to 1 g of MS222 in 1 L of seawater) for 10 min. Euthanized fish were dissected and fish muscle, gills, liver and brain tissues were collected. Each tissue (approximately 100 mg of muscle, gills and liver, and about 40 mg of brain) was individually homogenized in ice-cold conditions with 1.0 mL of phosphate buffered

saline (PBS; 140 mM NaCl, 3 mM KCl, 10 mM KH_2PO_4, pH = 7.40 ± 0.02; reagents from Sigma-Aldrich, Germany), using an Ultra-Turrax® device (T25 digital, Ika, Germany). Crude homogenates were centrifuged in 1.5 mL microtubes for 15 min at 10.000*g* and 4 °C. Supernatants were then transferred to new microtubes, immediately frozen and kept at − 80 °C until further analyses.

2.4. Integrated ecotoxicological response of *A. regius*

To assess fish tissue responses to VFX, warming and acidification exposure, eight ecotoxicological biomarkers (of exposure and/or effect) were selected, each corresponding to distinct biological endpoints:

i) Antioxidant defences – catalase activity [CAT; spectrophotometric enzymatic assay adapted from Johansson and Borg (1988)], superoxide dismutase activity [SOD; spectrophotometric enzymatic assay adapted from Sun et al. (1988)] and glutathione S-transferases activity [GST; spectrophotometric enzymatic assay adapted from Habig et al. (1974)];
ii) Cellular damage – Lipid peroxidation [(LPO); measured as the total malondialdehyde (MDA) content through the thiobarbituric acid test, adapted from Uchiyama and Mihara (1978)];
iii) Protein chaperoning/Heat shock response – HSP70/HSC70 content [determined through an enzyme-linked immunosorbent assay (ELISA), based on the methodology described by Njemini et al. (2005)];
iv) Protein degradation/DNA repair – Ubiquitin content [Ub; determined through the ELISA methodology, as described by Madeira et al. (2014)];
v) Reproduction/Endocrine disruption – Vitellogenin liver content [VTG; determined through the ELISA methodology based on the methodology described by Denslow et al. (1999)];
vi) Neurotoxicity – Acetylcholinesterase activity [AChE; spectrophotometric enzymatic assay adapted from Ellman et al. (1961)].

These biochemical biomarkers have been widely employed in ecotoxicological studies, being considered as reliable and suitable to assess the effects of xenobiotics exposure, including antidepressants (e.g. Fong and Ford, 2014; Rodrigues et al., 2014; Ding et al., 2017), as well as of climate change-related effects (e.g. Rosa et al., 2016; Maulvault et al., 2017, 2018c; Sampaio et al., 2018). To normalize the results of each biomarker (i.e. results expressed in mg of protein), total protein levels were also quantified in each sample according to the Bradford assay (Bradford, 1976). Furthermore, to facilitate data consultation and interpretation, biomarker results are presented throughout as U mg^{-1} protein, with the exception of SOD for which values were presented as % of inhibition (all biomarker units can be consulted in Appendix B, *Methodologies*). All biomarker assays were carried out using reagents of pro analysis grade or higher, as well as 96-well microplates from Nunc-Roskilde (Denmark) and a microplate reader (Multiskan Go 1510, ThermoFisher Scientific, USA). Further details regarding the methodologies used to determine tissue ecotoxicological responses are available in Appendix B, *Methodologies*. Each sample was analysed in triplicate.

In order to integrate the various ecotoxicological responses, the integrated multi-biomarker response (IBR) was calculated for each treatment and tissue, according to the methodology proposed by Beliaeff and Burgeot (2002), later modified by Guerlet et al. (2010). Further details regarding the IBRs calculations are presented in Appendix B, *Methodologies*. As IBR compares biomarker responses of organisms exposed to stressors with those of animals under control conditions, in general, lower biomarker scores (and, thus, lower IBR index values) indicate a better health status (higher animal fitness), whereas higher scores usually indicate that organisms are in a poorer physiological condition (i.e. stressed; e.g. Ferreira et al., 2015; Madeira et al., 2016, 2018; Maulvault et al., 2018c). To compare *A. regius* physiological state from a whole organism perspective, the average IBR value for each treatment (using values from all tissues) was also calculated (Madeira et al., 2016). Star plots and IBR calculations were performed using Microsoft Excel software.

2.5. Statistical analyses

As standard procedure, data were first tested for normality and homoscedasticity through Kolmogorov–Smirnov and Levene tests, respectively. Data were log or square-rooted transformed, whenever at least one of these assumptions was not verified. To evaluate the presence of significant differences between treatments in biomarker levels, nested factorial ANOVAs were carried out, using replicate tank as nesting factor, and tissue (brain, liver and muscle) and/or treatment as variables. Moreover, to determine the existence of significant differences in IBRs between treatments (all tissues combined) and tissues (all treatments combined), a simple one-way ANOVA analysis was performed instead. After performing the ANOVA analyses, post-hoc Tukey HSD tests were conducted to identify significant differences. Statistical analyses were performed at a significance level of 0.050, using STATISTICA™ software (Version 7.0, StatSoft Inc., USA).

3. Results

3.1. Biomarker tissue levels

Tissue biomarker responses in *A. regius* exposed to the different treatments are shown in Figs. 2–4 (biomarker values, i.e. activity/concentration, in CTR treatment can also be consulted in Appendix A, Table A3). Diminished CAT activity was generally observed in the muscle of fish exposed to VFX (regardless of exposure route), acidification and/or warming in relation to CTR treatment (i.e. decreases ranging from 31% in Acid and Warm treatments up to 68% in Acid + Warm + VFX-water treatment; $p < 0.050$), with the exception of VFX-water and Acid + Warm + VFX-feed treatments in which no significant changes were observed ($p > 0.050$; Figs. 2A and Table 1). In contrast, CAT activity was significantly enhanced by VFX exposure in fish liver (i.e. VFX-feed and VFX-water treatments) and by warming in brain, regardless of pCO_2 conditions and VFX exposure (i.e. CAT activity Warm, Warm + VFX-feed, Acid + Warm, Acid + Warm + VFX-feed and Acid + Warm + VFX-water treatments; $p < 0.050$; Fig. 2C-D and Table 1). As for fish gills, only VFX-feed and Acid + Warm + VFX-water treatments significantly affected CAT activity in this tissue, the first treatment inhibiting this enzyme's activity (52% decrease; $p < 0.050$), and the second enhancing it (56%; $p < 0.050$; Fig. 2B and Table 1). In general, all stressors diminished SOD activity at least in one tissue, yet, such effect was particularly notorious in Acid + Warm + VFX-water treatment, as all studied tissues showed significantly higher inhibition compared to CTR treatment (maximum SOD inhibition obtained in fish brain, corresponding to 21% decrease in relation to CTR treatment; $p < 0.050$; Fig. 2E-H and Table 1). Except for acidification alone, all stressors significantly increased GST activity in fish muscle, with the highest value being found in treatments simulating VFX water exposure (i.e. > 100 increase in VFX-water and Acid + Warm + VFX-water treatments; $p < 0.050$; Fig. 2I and Table 1). Similarly, GST brain activity was also significantly enhanced by warming alone or combined with the other two stressors (though the highest value was found in Warm treatment, corresponding to an increase of > 100% in relation to CTR treatment; $p < 0.050$), as well as by the combination of acidification plus VFX via feed (i.e. 93% increase in Acid + VFX-feed treatment in relation to CTR treatment; $p < 0.050$; Fig. 2L and Table 1). In contrast, a significant inhibition of this enzyme's activity was observed in the liver of fish from VFX-water (60%), Acid (63%), Acid + VFX-feed (36%), Warm (26%) and Acid + Warm (57%) treatments ($p < 0.050$; Fig. 2K and Table 1). All stressors (with the exception of VFX-feed exposure alone) and their interactions significantly increased total MDA concentrations compared to CTR treatment, with tissue LPO being particularly significant in fish gills and brain regardless of treatment (i.e. MDA gill content increased ~100%

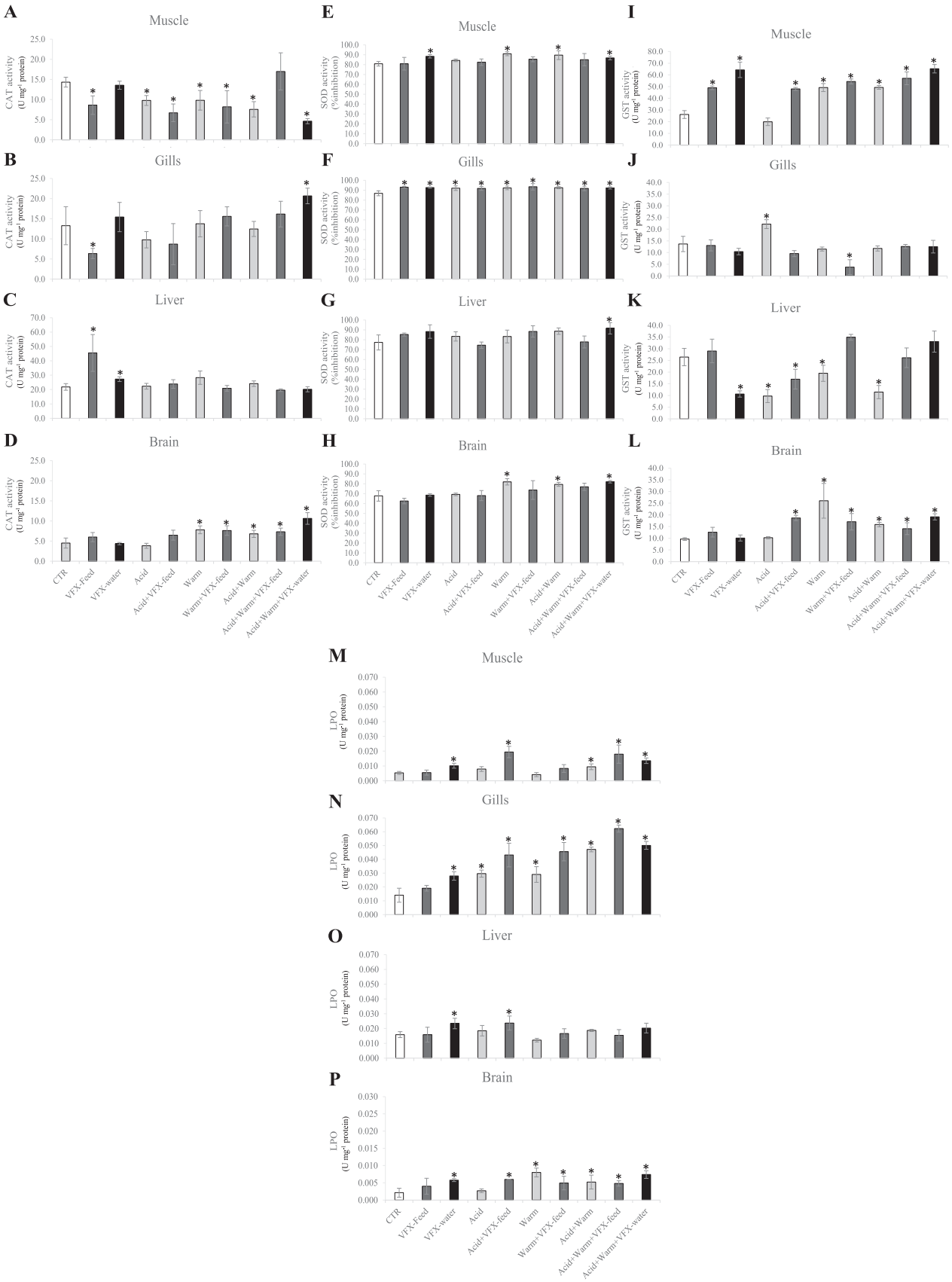

Fig. 2. Antioxidant enzymes activity (GST, U mg^{-1} protein; CAT, U mg^{-1} protein; SOD, % inhibition) and lipid peroxidation (LPO, U mg^{-1} protein) in muscle (A, E, I, M), gills (B, F, J; N), liver (C, G, K; O) and brain (D, H, L, P) in *A. regius* (average ± standard deviation; $n = 6$) after 28 days of exposure to the different experimental conditions. Asterisk indicate significant differences between CTR and the remaining treatments ($p < 0.05$). Abbreviations: CTR – reference temperature and pH conditions (i.e. T = 19 °C and pH = 8.0 units); VFX-feed – VFX exposure via feed; VFX-water – VFX exposure via water; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 °C).

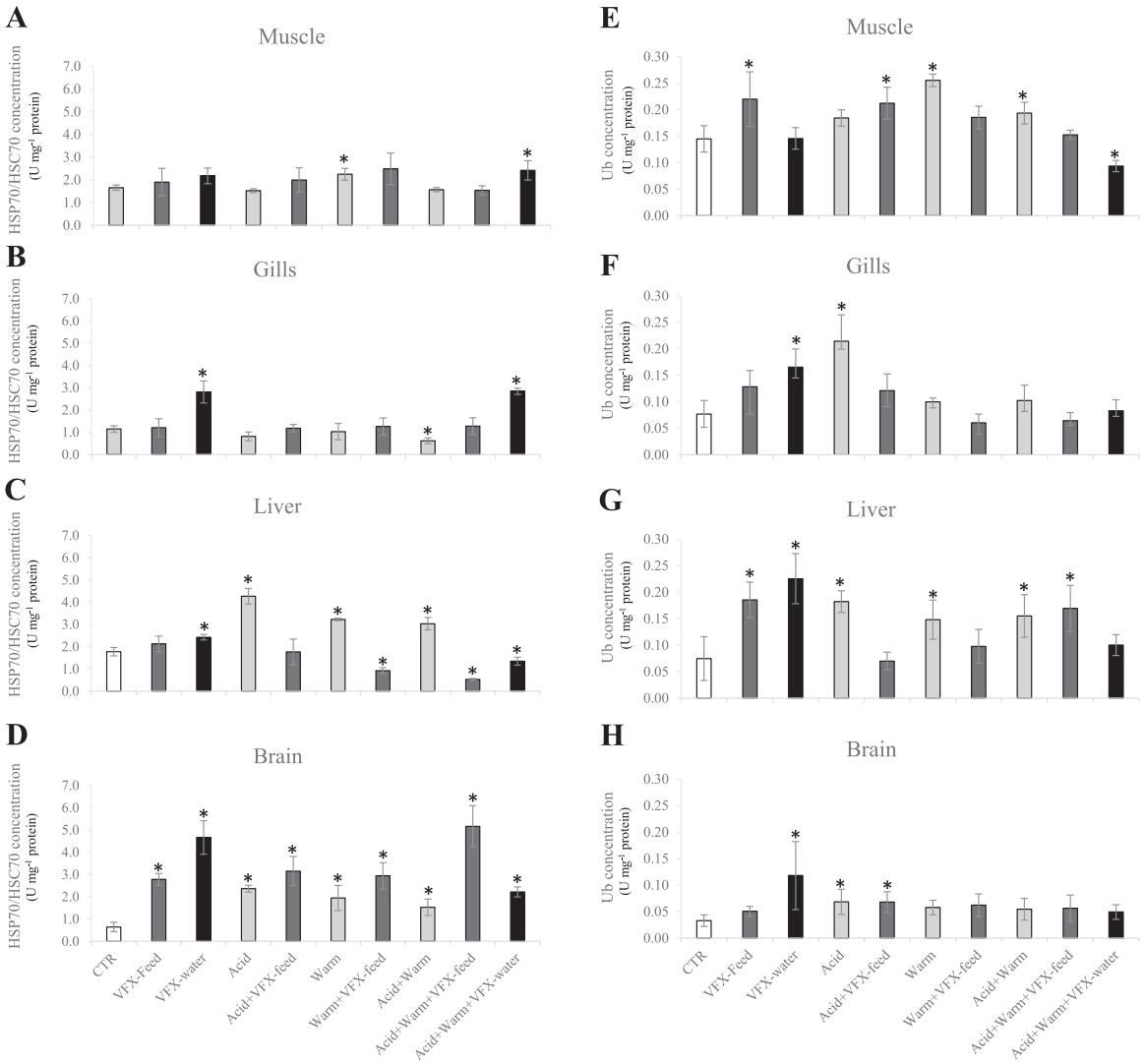

Fig. 3. Heat shock proteins (HSP70/HSC70; A-D) and total ubiquitin (Ub; E-H) concentrations (U mg^{-1} protein; average ± standard deviation; $n = 6$) in muscle (A, E), gills (B, F), liver (C, G) and brain (D, H) in *A. regius* after 28 days of exposure to the different experimental conditions. Asterisk indicate significant differences between CTR and the remaining treatments ($p < 0.05$). Abbreviations: CTR – reference temperature and pH conditions (i.e. T = 19 °C and pH = 8.0 units); VFX-feed – VFX exposure via feed; VFX-water – VFX exposure via water; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 °C).

in all treatments, with the exception of VFX-feed treatment; $p < 0.050$; Fig. 2M-P and Table 1). Noteworthy, the highest LPO value was found in fish gills from Acid + Warm + VFX-feed treatment (i.e. 0.062 ± 0.003 U mg protein^{-1}), corresponding to an average increase of 345% in relation to CTR treatment (Fig. 2N and Table 1).

HSP70HSC70 and Ub contents are presented in Fig. 3. Protein chaperoning and degradation was overall induced by the exposure to the three stressors (alone or combined), with the brain of fish from Acid + Warm + VFX-feed treatment revealing the highest HSP70/HSC70 content (i.e. 5.16 ± 0.92 μg mg protein^{-1}, i.e. 706% increase in relation to CTR; Fig. 3A-D and Table 1), whereas higher Ub contents were found when stressors acted individually (muscle: 0.26 ± 0.01 μg mg protein^{-1} in Warm treatment, i.e. 76% increase; gills: 0.21 ± 0.05 μg mg protein^{-1} in Acid treatment, i.e. 180% increase; liver and brain: 0.23 ± 0.05 μg mg protein^{-1} and 0.12 ± 0.06 μg mg protein^{-1}, respectively, i.e. 202% and 254% increases, respectively, both in VFX-water treatment; $p < 0.050$; Fig. 3E-H and Table 1). Noteworthy, in fish muscle, HSP70/HSC70 synthesis was only significantly induced in Warm (36% increase; $p < 0.050$) and Acid + Warm + VFX-water (46% increase; $p < 0.050$) treatments (Fig. 3A-D and Table 1). Yet, some exceptions were also observed, i.e. significant HSP70/HSC70 synthesis inhibition was observed when warming was combined with acidification (i.e. 46% reduction in gills from Acid + Warm treatment) and/or VFX via feed or water (i.e. 48%, 71% and 24% reduction in liver from Warm + VFX-feed, Acid + Warm + VFX-feed and Acid + Warm + VFX-water, respectively; $p < 0.050$; Fig. 3A-D and Table 1). Moreover, Ub synthesis was also impaired in fish muscle Acid + Warm + VFX-water treatment (i.e. 35% reduction in VFX relation to CTR treatment; $p < 0.050$; Fig. 3E and Table 1.

VTG liver content and AChE brain activity are shown in Fig. 4. VTG contents in the liver of *A. regius* were significantly increased by warming and VFX exposure via feed acting alone or combined (i.e. 47% increase in Warm treatment and 122% increase in VFX-feed and Warm + VFX-feed treatments in relation to CTR treatment; $p < 0.050$), whereas acidification alone or combined with warming and VFX via water significantly inhibited the production of this protein (i.e. 57% and 54% in Acid and Acid + Warm + VFX-water treatments, respectively, in relation to CTR treatment; $p < 0.050$; Fig. 4A and Table 1). All treatments significantly enhanced AChE activity in the brain, apart from VFX-water treatment, though it is worth mentioning that warming alone or combined with acidification and/or VFX-water exposure yielded the highest enzyme activity (i.e. 168%, 173% and 159% increases in Warm, Acid + Warm and Acid + Warm + VFX-water treatments, respectively; Fig. 4B and Table 1).

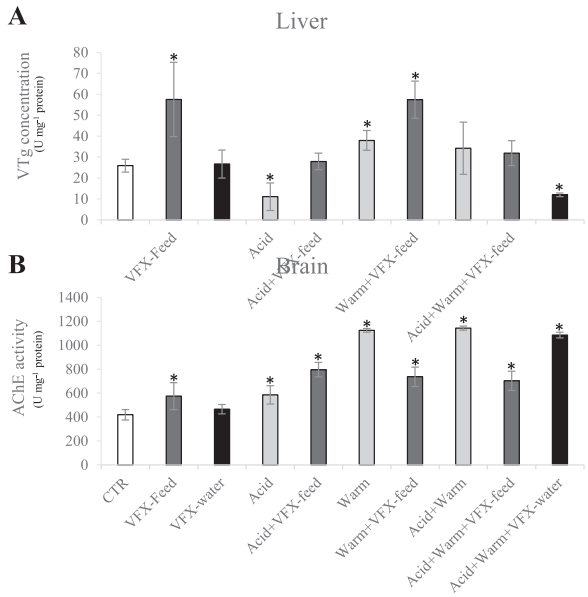

Fig. 4. Vitellogenin liver concentration (VTG; A) and acetylcholinesterase brain activity (AChE, B) in *A. regius* (U mg^{-1} protein; average ± standard deviation; $n = 6$) after 28 days of exposure to the different experimental conditions. Asterisk indicate significant differences between CTR and the remaining treatments ($p < 0.05$). Abbreviations: CTR – reference temperature and pH conditions (i.e. T = 19 °C and pH = 8.0 units); VFX-feed – VFX exposure via feed; VFX-water – VFX exposure via water; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 °C).

3.2. Integrated multi-biomarker responses (IBRs)

IBR index values for each tissue and treatment and the corresponding starplots are shown in Fig. 5 (individual scores of each biomarker in the different fish tissues and treatments can also be consulted shown in Appendix A, Table A4). In general, CTR samples presented lower biomarker scores than those obtained in the remaining treatments (differing in ≥ 0.5 units) and, thus, lower total IBR indexes were always found in this treatment (with the exception of muscle from Acid treatment, which revealed a value similar to CTR samples), regardless of tissue (Fig. 5 and Appendix A, Table A4). Differences between CTR and the other treatments were further confirmed through the *One-Way ANOVA* analysis combining the total IBR index of all tissues (*One-way ANOVA* results*: MS* = 85.47; $F = 5.98$ and $p < 0.001$; Fig. 5A). In contrast, no significant differences among tissues (all treatments combined) were observed (*One-way ANOVA* results*: MS* = 27.75; $F = 0.69$ and $p = 0.563$; Fig. 5A).

Looking at the variations according to exposure route, VFX-feed treatment revealed slightly higher total IBR index values than VFX-water treatment in fish muscle (i.e. 16 against 15, respectively) and liver (i.e. 22 against 19, respectively), whereas the opposite trend was observed in the brain (i.e. 11 against 20, respectively; Fig. 5A). In fish gills, similar values were yielded by the two VFX exposure routes (i.e. around 21; Fig. 5A). Combining the responses of all tissues, higher mean IBR index value was obtained in VFX-water treatment (i.e. 19) compared to VFX-feed treatment (i.e. 17; Fig. 5A).

Regarding the effect of abiotic stressors (acidification and warming acting separately), in fish muscle, acidification acting alone yielded the lowest IBR value (i.e. IBR = 6), while similar values were found in Acid + VFX-feed, Warm and Warm + VFX-feed treatments (i.e. IBRs around 15; Fig. 5A). In contrast, fish exposed to warming alone presented not only a lower IBR value in fish liver (i.e. IBR = 15) compared to the ones obtained in Acid, Acid + VFX-feed and Warm + VFX-feed treatments (i.e. IBRs = 18, 18 and 21, respectively), but also the highest IBR in the brain (i.e. IBR = 24; Fig. 5A). Interestingly, gills of non-contaminated fish exposed to acidification or warming showed higher IBR index values than those co-exposed to VFX via feed (i.e. 18.6 in Acid treatment against and 10.3 in Acid + VFX-feed treatment, and 13.7 in Warm treatment against and 9.8 in Warm + VFX-feed treatment; Fig. 5A). As for the combination of acidification plus warming, higher IBR index values were always obtained when VFX exposure was also added to the equation, i.e. Acid + Warm treatment always present lower IBR index values (mean IBR = 13) than Acid + Warm + VFX-water and Acid + Warm + VFX-feed treatments (Fig. 2). Moreover, Acid + Warm + VFX-water treatment also revealed higher values than Acid + Warm + VFX-feed treatment with the exception of fish muscle (i.e. 19), therefore yielding the highest mean IBR index out of all treatments (i.e. mean IBR all tissues combined = 21; Fig. 5A).

Concerning the contribution of each analysed biomarker to the total IBR index value, differential patterns were observed according to tissue and treatment (Fig. 5B-Q and Appendix A, Table A4). Starting with the CTR treatment, the most responsive biomarker was SOD (i.e. SOD scores within the four highest values in all tissues) followed, in this order, by CAT (in muscle, gills and brain), GST (in gills and liver) and LPO (in liver and brain). Conversely, lower scores were always attributed to HSP70/HSC70, VTG and AChE (Fig. 5B-Q and Appendix A, Table A4).

As for treatments simulating the exposure to stressors, SOD also consistently presented high scores, as did CAT in fish gills (except in Acid and Acid + VFX-feed, in which the IBR index value was overruled by GST instead) and LPO in the brain (Fig. 5B-Q and Appendix A, Table A4). In general, Ub and VTG provided important contributions to the total IBR indexes in the muscle and liver of fish exposed to acidification and/or VFX via feed (i.e. VFX-feed, Acid and Acid + VFX-feed treatments; (Fig. 5B-Q and Appendix A, Table A4). In addition, these two biomarkers also played an important role in the liver of fish from Warm + VFX-feed and Acid + Warm + VFX-water treatments (Fig. 5B-Q and Appendix A, Table A4). On the other hand, fish muscle (except in Warm + VFX-feed and Acid + Warm + VFX-feed treatments) and liver (all treatments) exposed to warmer seawater temperatures usually exhibited high scores of HSP70/HSC70, regardless of pCO_2 levels (Fig. 5B-Q and Appendix A, Table A4). Finally, it is also worth mentioning that fish brain also denoted important contributions of AChE in Acid + Warm and Acid + Warm + VFX-water treatments, but not in Acid + Warm + VFX-feed treatment, in which higher scores were rather attributed to HSP70/HSC70 (Fig. 5B-Q and Appendix A, Table A4).

4. Discussion

4.1. Differential biomarker responses to VFX exposure route

Studies assessing the effects of exposure route on contaminants' uptake and toxicity to marine biota are still scarce and, so far, to the best of our knowledge, most studies on the ecotoxicological implications of antidepressants have only focused on water exposure (e.g. Brooks, 2014; Fong and Ford, 2014; Chen et al., 2018; Pan et al., 2018). Although PPCPs are, in general, assumed to be mostly uptaken from water by marine biota, our previous study with *A. regius* constituted a proof of concept that, indeed, dietary exposure can substantially contribute to the total contaminant body burden in fish, even if to a lower extent compared to water exposure, with fish liver being the primary organ for VFX bioaccumulation, regardless of exposure route (Maulvault et al., 2018b). Here, we provide relevant and innovative data that point out that: i) distinct tissue biochemical responses (i.e. no effects or up/down-regulations) are triggered when different pathways of antidepressants' exposure take place (i.e. water and diet); and ii) such differential tissue responses are not necessarily linked to the corresponding VFX tissue burdens elicited by the two VFX exposure routes (i.e. higher VFX tissue concentrations elicited by water exposure; Maulvault et al., 2018a); in fact, it seems that the exposure pathway influenced VFX toxicity, as well as tissue susceptibility to this compound.

Fish antioxidant mechanisms were altered by both VFX exposure routes, namely through the enhancement of CAT (in the liver) and GST (in

Table 1
Percentages of change (%) induced by VFX (via feed or water) exposure, acidification and warming in relation to the CTR treatment, as well as, statistical comparisons (Tukey HSD test) between CTR and the other treatments (i.e. *p-values*; $n = 6$ individuals analysed per treatment). "↑" before the value indicates a significant increase compared to values found in CTR treatment, whereas "↓"indicates a significant decrease ($p < 0.05$). Abbreviations: CAT – catalase activity; SOD – superoxide dismutase activity; GST – glutathione S-transferase activity; LPO - lipid peroxidation measured as the MDA concentration; HSP70/HSC70 - heat shock proteins concentration; Ub - total ubiquitin concentration; VTG – vitellogenin concentration; AChE – acetylcholinesterase activity; NS – No significant alteration ($p > 0.05$) compared to the CTR treatment; VFX-feed – VFX exposure via feed; VFX-water – VFX exposure via water; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 °C). *(Continued)*.

		CAT		SOD		GST		LPO		HSP70/HSC70		Ub		VTG		AChE	
		%	*p-value*	%	*p-value*	%	*p-value*	%	*p-value*	%	*p-value*	%	*p-value*	%	*p-value*	%	*p-value*
VFX-Feed	**Muscle**	**↓39.8**	0.024	NS	> 0.050	**↑87.3**	< 0.001	NS	> 0.050	NS	> 0.050	**↑51.8**	< 0.001	–		–	–
	Gills	**↓52.1**	0.040	**↓7.3**	< 0.001	NS	> 0.050	NS	> 0.050	NS	> 0.050	NS	> 0.050	–		–	–
	Liver	**↑108.3**	< 0.001	NS	> 0.050	NS	> 0.050	NS	> 0.050	NS	> 0.050	**↑148.8**	0.001	**↑122.1**	< 0.001	–	–
	Brain	NS	> 0.050	NS	> 0.050	NS	> 0.050	NS	> 0.050	**↑334.1**	< 0.001	NS	> 0.050	–		**↑37.2**	0.004
VFX-water	**Muscle**	NS	> 0.050	**↓9.3**	0.011	**↑146.1**	< 0.001	**↑90.3**	0.008	NS	> 0.050	NS	> 0.050	–		–	–
	Gills	NS	> 0.050	**↓6.8**	< 0.001	NS	> 0.050	**↑99.5**	0.003	**↑145.3**	< 0.001	**↑115.6**	0.001	–		–	–
	Liver	**↑25.0**	0.032	NS	> 0.050	**↓59.8**	< 0.001	**↑47.6**	0.032	**↑36.5**	0.020	**↑202.4**	< 0.001	NS	> 0.050	–	–
	Brain	NS	> 0.050	NS	> 0.050	NS	> 0.050	**↑169.9**	0.006	**↑627.6**	< 0.001	**↑253.6**	0.008	–		NS	> 0.050
Acid	**Muscle**	**↓31.8**	0.033	NS	> 0.050	NS	> 0.050	NS	> 0.050	NS	> 0.050	NS	> 0.050	–		–	–
	Gills	NS	> 0.050	**↓6.1**	0.003	**↑62.1**	< 0.001	**↑11.6**	0.002	NS	> 0.050	**↑180.0**	< 0.001	–		–	–
	Liver	NS	> 0.050	NS	> 0.050	**↓63.2**	< 0.001	NS	> 0.050	**↑140.0**	< 0.001	**↑144.3**	0.001	**↓57.3**	0.004	–	–
	Brain	NS	> 0.050	NS	> 0.050	NS	> 0.050	NS	> 0.050	**↑268.5**	< 0.001	**↑104.8**	0.035	–		**↑39.7**	0.002
Acid + VFX-feed	**Muscle**	**↓53.2**	0.001	NS	> 0.050	**↑83.6**	< 0.001	**↑264.2**	< 0.001	NS	> 0.050	**↑46.5**	0.003	–		–	–
	Gills	NS	> 0.050	**↓5.8**	0.005	NS	> 0.050	**↑207.5**	< 0.001	NS	> 0.050	NS	> 0.050	–		–	–
	Liver	NS	> 0.050	NS	> 0.050	**↓35.8**	0.005	**↑49.3**	0.023	NS	> 0.050	NS	> 0.050	NS	> 0.050	–	–
	Brain	NS	> 0.050	NS	> 0.050	**↑93.3**	< 0.001	**↑179.6**	0.003	**↑391.9**	< 0.001	**↑104.0**	0.028	–		**↑89.9**	< 0.001
Warm	**Muscle**	**↓31.4**	0.042	**↓12.5**	0.004	**↑88.3**	< 0.001	NS	> 0.050	**↑35.9**	0.026	**↑76.3**	< 0.001	–		–	–
	Gills	NS	> 0.050	**↓6.4**	0.002	NS	> 0.050	**↑107.1**	0.002	NS	> 0.050	NS	> 0.050	–		–	–
	Liver	NS	> 0.050	NS	> 0.050	NS	> 0.050	NS	> 0.050	**↑81.4**	< 0.001	**↑101.8**	0.011	**↑46.6**	0.021	–	–
	Brain	**↑73.4**	0.001	**↓21.0**	< 0.001	**↑169.6**	< 0.001	**↑73.8**	< 0.001	**↑203.6**	0.013	NS	> 0.050	–		**↑168.3**	< 0.001
Warm + VFX-feed	**Muscle**	**↓42.8**	0.011	NS	> 0.050	**↑108.1**	< 0.001	NS	> 0.050	NS	> 0.050	NS	> 0.050	–		–	–
	Gills	NS	> 0.050	**↓7.8**	< 0.001	**↑72.4**	< 0.001	**↑224.9**	< 0.001	NS	> 0.050	NS	> 0.050	–		–	–
	Liver	NS	> 0.050	NS	> 0.050	NS	> 0.050	NS	> 0.050	**↓48.2**	< 0.001	NS	> 0.050	**↑122.0**	< 0.001	–	–
	Brain	**↑69.2**	0.001	NS	> 0.050	**↑76.5**	< 0.001	**↑130.3**	0.005	**↑358.6**	< 0.001	NS	> 0.050	–		**↑75.7**	< 0.001
Acid + Warm	**Muscle**	**↓47.1**	0.004	**↓10.8**	0.019	**↑88.6**	< 0.001	**↑77.1**	0.029	NS	> 0.050	**↑33.6**	0.025	–		–	–
	Gills	NS	> 0.050	**↓6.6**	< 0.001	NS	> 0.050	**↑236.3**	0.001	**↓46.2**	0.033	NS	> 0.050	–		–	–
	Liver	NS	> 0.050	NS	> 0.050	**↓56.7**	< 0.001	NS	> 0.050	**↑70.7**	< 0.001	**↑108.0**	0.023	NS	> 0.050	–	–
	Brain	**↑51.2**	0.036	**↓17.2**	0.004	**↑64.6**	< 0.001	**↑144.6**	0.031	**↑138.2**	< 0.001	NS	> 0.050	–		**↑172.9**	< 0.001
Acid + Warm + VFX-feed	**Muscle**	NS	> 0.050	NS	> 0.050	**↑118.5**	< 0.001	**↑236.6**	< 0.001	NS	> 0.050	NS	> 0.050	–		–	–
	Gills	NS	> 0.050	**↓5.8**	0.005	NS	> 0.050	**↑343.9**	< 0.001	NS	> 0.050	NS	> 0.050	–		–	–
	Liver	NS	> 0.050	NS	> 0.050	NS	> 0.050	NS	> 0.050	**↓70.5**	< 0.001	**↑127.2**	0.004	NS	> 0.050	–	–
	Brain	**↑62.6**	0.004	NS	> 0.050	**↑46.0**	0.011	**↑125.4**	< 0.001	**↑706.1**	< 0.001	NS	> 0.050	–		**↑67.7**	< 0.001
Acid + Warm + VFX-water	**Muscle**	**↓67.7**	< 0.001	**↓7.3**	0.016	**↑149.9**	< 0.001	**↑154.0**	< 0.001	**↑46.2**	0.015	**↓35.4**	0.049	–		–	–
	Gills	**↑55.7**	0.022	**↓6.4**	0.001	NS	> 0.050	**↑257.4**	< 0.001	**↑ > 100**	< 0.001	NS	> 0.050	–		–	–
	Liver	NS	> 0.050	**↓18.5**	0.006	NS	> 0.050	NS	> 0.050	**↓24.0**	0.026	NS	> 0.050	**↓53.6**	0.023	–	–
	Brain	**↑136**	< 0.001	**↓21.2**	< 0.001	**↑98.3**	< 0.001	**↑245.9**	< 0.001	**↑246.1**	0.001	NS	> 0.050	–		**↑159.0**	< 0.001

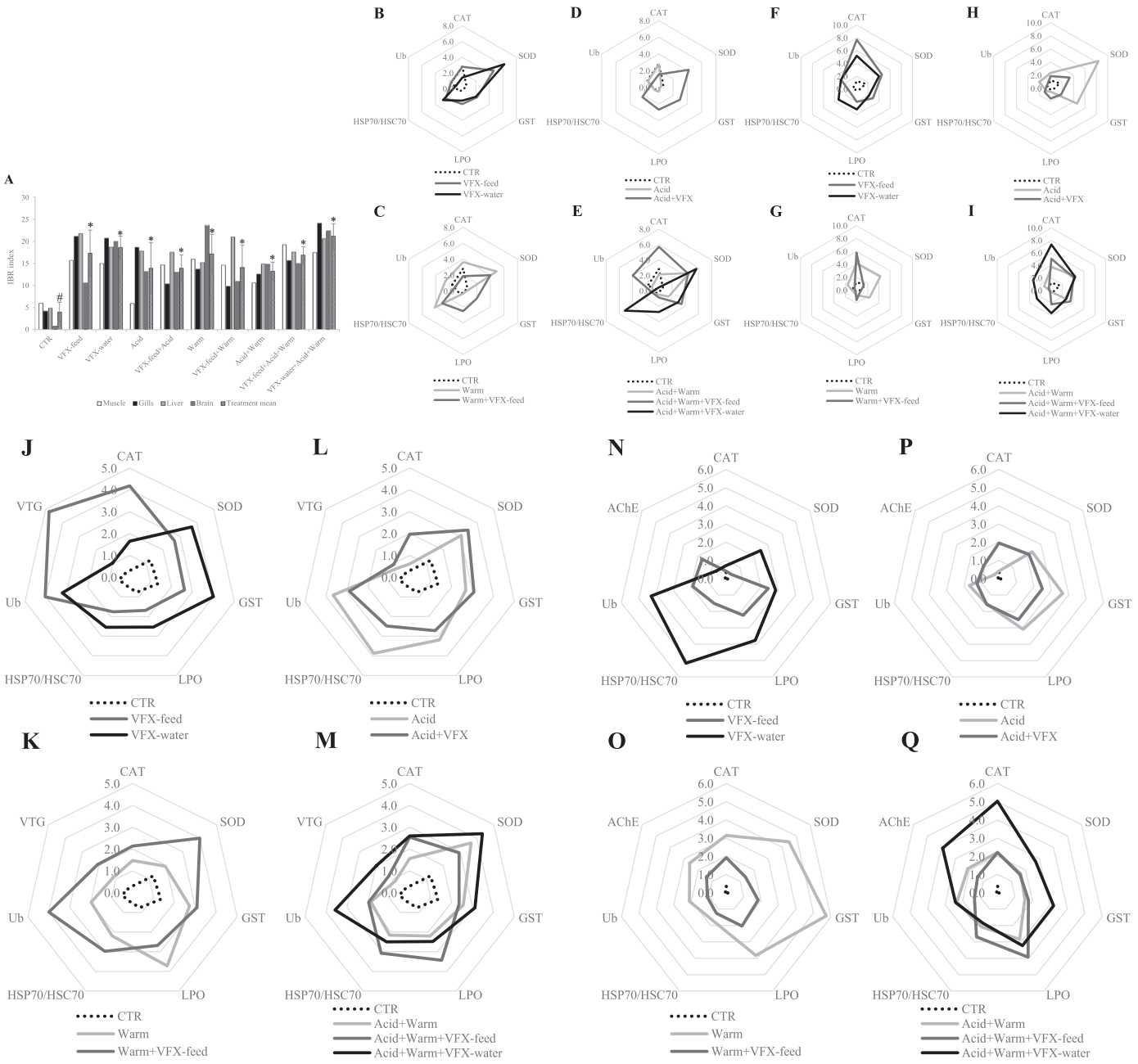

Fig. 5. IBR index values for each tissue/treatment (A) and star plots of biomarker scores in the different fish tissues (muscle: B – E; gills: F– I; liver: J-M; brain: N – Q). Different symbols (# or *) indicate significant differences between the mean IBR of each treatment (i.e. average IBR value of the 4 tissues analysed; $p < 0.05$). Abbreviations: CAT – catalase activity; SOD – superoxide dismutase activity; GST – glutathione S-transferase activity; LPO - lipid peroxidation, measured as MDA concentration; HSP70/HSC70 - heat shock proteins concentration; Ub - total ubiquitin concentration; VTG – vitellogenin concentration; AChE – acetylcholinesterase activity; CTR – reference temperature and pH conditions (i.e. T = 19 °C and pH = 8.0 units); VFX-feed – VFX exposure via feed; VFX-water – VFX exposure via water; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 °C).

the muscle) activities, a mechanism that is frequently activated to overcome the excessive formation of reactive oxygen species (ROS) induced by the exposure to stressors. On the other hand, muscle and gills showed diminished CAT activity (under VFX-feed exposure), as well as SOD activity (under both exposure routes). Such inhibition is likely associated with the fact that the antioxidant machinery was unable to compensate for an excessive production of substrate (i.e. superoxide radicals are converted into H_2O_2 by SOD, and then CAT converts H_2O_2 into H_2O and O_2) induced by VFX exposure (Gonzalez-Rey and Bebianno, 2014; Maulvault et al., 2018c). GST activity inhibition in the liver of fish exposed to VFX via water, but not in those exposed via feed, might also be related with the higher VFX concentration elicited by VFX water exposure (i.e. ~6810 μg kg^{-1} in VFX-water treatment against ~150 μg kg^{-1} in VFX-feed treatment; values previously reported in Maulvault et al., 2018a), which could have exhausted liver's mechanisms of xenobiotic detoxification, through a decreased formation of reduced glutathione (i.e. lower substrate to be used by GST; Gonzalez-Rey and Bebianno, 2011). Furthermore, such impairment of VFX's detoxification in fish liver may justify the increased formation of lipid peroxides (i.e. increased MDA concentration) found in all fish tissues from VFX-water treatment. Hence, results suggest that tissue antioxidant defences were able to prevent to some extent the oxidative stress induced by VFX exposure via feed, but not by VFX exposure via water, promoting cell damage in fish subjected to this treatment. In accordance with our findings, previous studies on aquatic species exposed to

antidepressants via water reported not only increased CAT and GST activities (in *Dreissena polymorpha*; Magni et al., 2017; *Danio rerio* and *Carassius auratus*; Pan et al., 2018), but also diminished SOD (in *Daphnia magna*; Ding et al., 2017) and GST (in the liver of *Pseudorasbora parva*; Chen et al., 2018) activities, both being accompanied by increased LPO (Ding et al., 2017; Chen et al., 2018). For instance, Chen et al. (2018) reported decreased CAT and GST activities alongside with increased LPO after long-term exposure to a high concentration of fluoxetine (200 $\mu g\,L^{-1}$). Yet, in this same study, a different pattern was observed under the lowest fluoxetine concentration (i.e. 50 $\mu g\,L^{-1}$), i.e. CAT activity increased after short-term exposure, but not in a long-term, while GST was not significantly affected (regardless of exposure duration; Chen et al., 2018). Hence, such concentration- and time-dependency in antidepressants mode of action may explain the differential effects elicited by the two pathways of VFX exposure in the present study.

The increased HSP70/HSC70 and Ub contents in fish exposed to VFX through both exposure routes is in agreement with the fact that the synthesis of these proteins is usually induced by the exposure to pollutants (e.g. Gravel and Vijayan, 2007; Horst et al., 2007; Ajima et al., 2018; Maulvault et al., 2018c), in order to prevent irreversible DNA damage, as the first mediates the repairing, refolding and elimination of damaged proteins (Sottile and Nadin, 2018), while the second is responsible for inactivating and tagging damaged proteins that are to be degraded by the proteasome (Jackson and Durocher, 2013). Yet, results suggest that VFX water exposure promoted cellular damage to a much higher extent compared to VFX feed exposure, given the substantial HSP70/HSC70 and Ub contents increase observed in fish gills, liver and brain from VFX-water treatment.

In opposition, VFX exposure via feed had a clear effect on AChE brain activity and VTG liver content (i.e. both increased), while no significant effects were elicited by VFX water exposure, despite the much higher VFX tissue concentrations observed in these tissues under VFX-water exposure (Maulvault et al., 2018a). The currently available literature has revealed some controversy in what concerns the effects of antidepressants on AChE activity in aquatic species, with some studies reporting its inhibition (e.g. Munari et al., 2014; Ding et al., 2017; Yang et al., 2018), and others describing an induction (e.g. Gonzalez-Rey and Bebianno, 2013; Rodrigues et al., 2014; Xie et al., 2015; Chen et al., 2018; Pan et al., 2018). Several authors have previously argued that antidepressants act on AChE in a time- (Gonzalez-Rey and Bebianno, 2013; Ding et al., 2017; Pan et al., 2018) and concentration-dependent (Munari et al., 2014; Rodrigues et al., 2014; Yang et al., 2018) manner, therefore, justifying the differential effects on AChE brain activity induced by the two VFX exposures routes simulated in our study. For instance, Munari et al. (2014) reported AChE activity inhibition in clams (*Venerupis philippinarum*) exposed to 1 and 5 $\mu g\,L^{-1}$ of fluoxetine, but not in clams exposed to 25, 125 and 625 $\mu g\,L^{-1}$. Conversely, crab specimens *Carcinus maenas* exposed to sertraline-contaminated seawater exhibited an up-regulation of AChE activity (in muscle) under a low compound concentration (0.05 $\mu g\,L^{-1}$) and, concomitantly, a down-regulation under a high concentration (5 $\mu g\,L^{-1}$; Rodrigues et al., 2014). In the present study, the increased AChE activity elicited by VFX-feed exposure (acting alone) can have three potential justifications: i) VFX-feed exposure could have promoted brain cell apoptosis, causing the release of AChE from brain cells (Zhang et al., 2002; Gonzalez-Rey and Bebianno, 2013, 2014); ii) similar to what has been reported for human subjects, exposure to stressful conditions can increase the synthesis of AChE splicing variants (e.g. AChE-R; Lionetto et al., 2013); and iii) as previously reported for other antidepressants [e.g. increased VTG1 gene expression in brain and gonads of *Danio rerio* following the exposure to mianserin (van der Ven et al., 2006); inhibition VTG-like proteins (measured indirectly as alkali-labile phosphates measurement in *Mytillus galloprovincialis* exposed to fluoxetine (Gonzalez-Rey and Bebianno, 2014)], VFX (at the tissue and plasma concentrations elicited by feed exposure) might have had an estrogenic effect through the disturbance of the hypothalamo–pituitary–gonadal (HPG) axis, causing increased estrogen and VTG-like protein levels which, in turn, modulated the cholinergic system, including AChE activity (van der Ven et al., 2006; Gonzalez-Rey and Bebianno, 2014; Oliveira et al., 2015). The second and/or third hypothesises seem more plausible, since increased brain cell apoptosis would also presume increased LPO and altered chaperone and ubiquitin contents, which was not the case in VFX-feed treatment. Moreover, the third argument also matches the induction of VTG synthesis observed in the liver of VFX-feed exposed fish. Yet, further research on fish neuroendocrine responses to antidepressants (particularly, studies assessing AChE splicing forms, neurotransmitters, as well as sexual and thyroid hormone levels) are required in the future to confirm these hypothesises.

4.2. Interactive effects of warming and acidification

Overall, results evidenced that both warming and acidification strongly influenced fish coping mechanisms to the presence of antidepressants, resulting in either an enhancement of tissue ecotoxicological responses or in their attenuation/reversion. Such differential tissue responses are likely related to the fact that each tissue has distinct physiology, functioning and baseline levels of biochemical biomarkers, as they are composed by different cell types, and, therefore, may also respond differently to the interactive effects of environmental stressors.

Seawater warming and acidification have the potential to directly or indirectly influence marine species physiology, metabolism and overall fitness (Rosa et al., 2014, 2016, 2017; Madeira et al., 2016, 2018). Though many organisms have evolved to cope with daily or seasonal abiotic variations, their thresholds of physiological tolerance can be surpassed when the co-exposure to multiple stressors occurs at the same time (including exposure to pollutants), thus, compromising their fitness and ecological success (e.g. Rosa et al., 2016; Maulvault et al., 2017, 2018a; Sampaio et al., 2018). In this way, the additional physiological stress promoted by the co-exposure of VFX with abiotic stressors triggered cells antioxidant machinery (e.g. CAT in gills of fish exposed to VFX via water; CAT and SOD in brain of fish exposed to both VFX exposure routes, GST in muscle and brain of fish exposed to both VFX exposure routes). Although research on the interactive effects of climate change-related stressors is still in its infancy and, therefore, the available literature is limited, recent studies on marine biota have also reported a significant increase of CAT, SOD and GST activities when pollutants are co-exposed with higher temperatures and/or pCO_2 levels (e.g. Freitas et al., 2016; Maulvault et al., 2017, 2018c; Sampaio et al., 2018). Such enhancement occurs as a way to compensate the elevated formation of ROS, due to an intensive mitochondrial respiration along with diminished fish aerobic scope (and deprived oxygen supply to the different tissues; Heise et al., 2006; Pörtner and Peck, 2010). Nevertheless, cells' defences against low oxygen supplies and oxidative stress are time-dependent (Pörtner, 2002; Heise et al., 2006; Madeira et al., 2016, 2018), two different strategies may simultaneously take place under chronic stress conditions (such as the ones simulated in the present study, i.e. 28 days of exposure trial): i) after a certain period of acclimation, an organism may reach a state of internal homeostasis which enables to withstand stress and return to baseline levels (Madeira et al., 2018), i.e. not evidencing significant biomarker changes in relation to CTR, as occurred with CAT and SOD liver activities (all treatments, except Acid + Warm + VFX-water); or ii) when stress conditions are too severe and acclimation is no longer possible, animal metabolism can become depressed (and, consequently, protein synthesis is disturbed), translating into an inhibition of these enzymes' activities (Sokolova, 2013; Ferreira et al., 2015; Madeira et al., 2016; Maulvault et al., 2018c), e.g. as observed in CAT muscle activity (all treatments, except Acid + Warm + VFX-feed) and GST liver activity (in treatments simulating acidification, except when the three stressors were combined). Moreover, an attenuation (or even reversion) of tissue responses could also be observed, in some cases, when the co-exposure to different stressors occurred (e.g. SOD activity in fish muscle was

inhibited by increased temperature acting individually, but such inhibition was lowered by the co-exposure to acidification, whereas the co-exposure to VFX-feed did not induce significant alterations in relation to CTR treatment, regardless of pCO_2 levels). Similarly, in an earlier study using seabass (*Dicentrachus labrax*), diclofenac dietary exposure significantly decreased CAT activity (80% inhibition in relation to the control treatment), yet such inhibition was attenuated by the co-exposure to warming (63% inhibition) and/or acidification (57% inhibition; Maulvault et al., 2018c). The significant increase of LPO in all treatments (with the exception of VFX-feed) in relation to relation to CTR treatment indicated that, despite tissues' antioxidant defences were activated to some point (and extent), cell damage or even apoptosis occurred after 28 days of exposure to the studied stressors (particularly to acidification). Noteworthy, such tissue damage was particularly evident in fish gills (i.e. high concentrations of MDA in all treatments), and that could be related to the fact that gills are one of the most aerobic fish tissues (being responsible for fish breathing) and, therefore, are expected to be particularly sensitive to reduced oxygen levels caused by an impaired animal aerobic scope.

As previously mentioned, stressful environmental conditions can trigger the synthesis of molecular chaperones, such as HSP70/HSC70, as way to repair reversible protein damage that antioxidant scavengers alone are not able to prevent (Madeira et al., 2017; Sottile and Nadin, 2018). Subsequently, when irreversible protein anomalies occur (i.e. molecular chaperoning mechanisms no longer can repair the cellular damage), the ubiquitin-proteosomal pathway is also initiated to signal and eliminate such proteins (Jackson and Durocher, 2013; Madeira et al., 2017). Yet, since protein synthesis is an extremely demanding process from the energetic point of view (requiring over 50% of an organism's total oxygen supply), both mechanisms of chaperoning and ubiquitination can be impaired when organisms fall into physiological collapse due to severe or long-lasting stress conditions (Hofmann and Somero, 1995; Gravel and Vijayan, 2007; Araújo et al., 2018; Maulvault et al., 2018c). Based on this background knowledge, the present results suggest that, overall, increased temperatures (i.e. Warm, Warm + VFX-feed, Acid + Warm, Acid + Warm + VFX-feed and Acid + Warm + VFX-water treatments) promoted reversible cellular damaged in fish brain, which was withstood by the induction of HSP70/HSC70. Although the diminished HSP70/HSC70 and unchanged Ub contents in fish liver from Warm + VFX-feed and Acid + Warm + VFX-feed treatments could indicate, at a first glance, impaired cellular responses (Araújo et al., 2018), the trends of antioxidant enzymes and LPO in these treatments indicate that the physiological state of fish liver was favoured by warming, possibly due to an enhanced animal metabolism (and, thus, enzymatic activity and protein synthesis) which could have counteracted the negative effects of VFX feed exposure and/or acidification. In contrast, the simultaneously co-exposure to VFX via water, acidification and warming seemed to have caused severe impairments in tissues' protective mechanisms, as revealed by the increased chaperone synthesis in fish muscle, gills and brain, together with the inhibition of these proteins in the liver and of Ub in the muscle. Such depressed physiological stated could be likely attributed to the higher VFX tissue burdens elicited by VFX water exposure (in relation to VFX feed exposure, regardless of abiotic stressors; Maulvault et al., 2018a), which deteriorated fish aerobic scope and impaired protein synthesis (Falfushynska et al., 2014; Madeira et al., 2017).

Seawater abiotic conditions, such as temperature and pH, play a key role on fish reproduction, determining the success of oocyte maturation, ovulation and spawning (e.g. Brown et al., 2006; Arantes et al., 2011; Milazzo et al., 2016). In line with this, both climate change-related stressors affected *A. regius* neuroendocrine response regardless of VFX exposure, with warming being responsible for an induction of VTG production and AChE activity, whereas acidification not only inhibited VTG synthesis but also increased AChE activity, though to lower extent compared to warming. This overstimulation of fish neuroendocrine responses in treatments simulating warming (regardless of VFX exposure) is in accordance with previous findings on the effects of temperature and chemical pollutants (endocrine disrupting compounds; Chandra et al., 2012; Maulvault et al., 2018c; Shappell et al., 2018). For instance, Chandra et al. (2012) reported increased VTG1 mRNA gene expression in *Fundulus heteroclitus* male specimens exposed to the combination of 17-ethynylestradiol and increased seawater temperature (26 °C). As hypothesized by these authors, such enhancement is certainly related to the exacerbation of fish metabolic rates (and, consequently, enzyme activities) at warmer temperatures. Yet, it should be noted that drastic temperature variations (i.e. outside species' physiological thresholds) have also been associated with inhibitory reproductive effects in teleost species (Pankhurst and Munday, 2011; Miranda et al., 2013). Therefore, the present results suggest that, despite the elicited changes, a temperature increase of + 5 °C may still fall within the reproductive thermal window of *A. regius*. The present results are also consistent with the trends observed in our previous studies with *D. labrax* (Maulvault et al., 2018c) and *Diplodus sargus* (Maulvault et al., 2018d), as increased pCO_2 levels evidenced an anti-estrogenic effect (i.e. VTG inhibition) and cholinergic modulation, possibly due to a disturbance of brain ionic homeostasis which, in turn, impaired neurotransmission and hormone synthesis (Pankhurst and Munday, 2011; Nilsson et al., 2012; Kwong et al., 2014; Heuer et al., 2016). Yet, such effects seemed to have been attenuated by warming and, to a lower extent, by VFX feed exposure, but not by VFX water exposure, once again, pointing out differential tissue responses to VFX exposure route. Since, so far, research on the reproductive effects of acidification (as well as warming) has been mostly focused on sensory and behavioural aspects (e.g. Nilsson et al., 2012; Munday et al., 2014; Maulvault et al., 2018b), further studies in this direction are still required to better understand the biochemical processes involved in fish neuroendocrine responses to climate change-related stressors.

4.3. Using IBRs to estimate the overall fish fitness

To fully understand the effects of environmental stressors can become a challenging task, especially, when different tissues with distinct sensitivity and biomarker responses are analysed, as well as when multiple stressors interact with each other. Hence, IBRs constitute an innovative and practical tool that enables a qualitative assessment of the overall fitness of organisms, as well as comparisons among different stressors according to their magnitude of severity (e.g. Kamel et al., 2014; Ferreira et al., 2015; Madeira et al., 2016, 2018; Maulvault et al., 2018c). The use of this tool also evidences the sensitivity of each biomarker and tissue to respond to a specific stressor (Madeira et al., 2016, 2018; Maulvault et al., 2018a).

Starting with biomarkers' sensitivity to the studied stressors, the fact that SOD and LPO consistently yielded high scores, regardless of tissue and treatment, pointed out to a lack of specificity when responding to the three studied stressors acting individually or to their interactions. Conversely, in accordance with previous findings (Madeira et al., 2016, 2018), HSP70/HSC70 content proved to be a sensitive biomarker of thermal stress (in fish muscle, liver and brain), despite its induction was reduced or even inhibited by the co-exposure to VFX or acidification. Even though no previous IBR studies specifically focusing on the effects of acidification and antidepressants exposure were found, the present data showed an overall good reactivity of Ub and liver VTG content to acidification, thus, confirming the cytotoxic and anti-estrogenic potential of increased pCO_2 levels (Maulvault et al., 2018a).

Using IBRs to compare stressors severity, as far as the effect of VFX exposure route is concerned, results evidenced that fish muscle, gills and liver were more susceptible to VFX exposure via feed (i.e. higher IBR) than to VFX water exposure, and such susceptibility was mostly attributed to the remarkable changes induced by this exposure pathway in CAT activity, as well as in Ub and VTG contents. Yet, the remarkable increase in LPO, protein chaperoning and degradation in fish exposed to VFX via water resulted in a much higher degree of stress in fish brain (i.e. higher IBR). The substantial increase of these biomarkers' scores also translated into a poorer physiological state of water exposed fish (i.e. higher average IBR index, combining the integrated responses of all

tissues) compared to those exposed via feed, a result that is in line with the differential VFX tissue concentrations elicited by both exposure routes (values previously reported in Maulvault et al., 2018a).

Regarding the interactive effects of climate change-related stressors, regardless of the remarkable effects on Ub and VTG liver contents, IBR results revealed that acidification had an overall negative impact on fish gills (promoting severe cell damage, according to the increased LPO and Ub contents), but its effects were less evident in the remaining tissues compared to those promoted by warming and/or VFX exposure. In contrast, warming alone proved to be particularly harmful to fish brain. This can be attributed to the enhanced tissue metabolic rates (i.e. increased CAT, GST and AChE activities and HSP70/HSC70 content) together with increased LPO. The comparatively lower IBR in fish brain from Warm + VFX-feed treatment was mainly due to the fact that VFX feed exposure attenuated biomarker changes induced by warming. The co-exposure to the three stressors (i.e. Acid + Warm + VFX-feed and Acid + Warm + VFX-water treatments) resulted, overall, in higher IBR index values, regardless of the lower VFX tissue concentrations elicited by these treatments (values presented in Maulvault et al., 2018a). These results reveal that, indeed, temperature and pCO_2 levels have a determinant role in fish fitness, especially when both abiotic stressors are combined. In line with these findings, the co-exposure to pollutants and abiotic stressors has been previously described to result in comparatively higher IBR values, leading to a poorer animal physiological state (Serafim et al., 2012; Kamel et al., 2014; Ács et al., 2016; Maulvault et al., 2018c). As occurred under normal temperature and pCO_2 conditions, IBR data also highlighted that a higher magnitude of stress was inferred when VFX exposure occurred via water (i.e. higher tissue IBR in Acid + Warm + VFX-water treatment compared to Acid + Warm + VFX-feed treatment, except in fish muscle).

As a final remark, it is also worth noting that the good responsiveness of AChE brain activity in fish co-exposed to the three stressors matched *A. regius* decreased exploratory behaviour and shoal cohesion observed in our earlier study (Maulvault et al., 2018c), pointing out severe neurological impairments, most likely linked to disrupted neurotransmission and/or brain cells' death.

5. Conclusions

In this study, we show in a comprehensive way that VFX toxicological attributes to marine fish species are strongly influenced by the uptake pathway, as well as by the surrounding abiotic conditions. Furthermore, our results highlighted the importance of analyzing multiple tissue responses as to have a broader view of fish ecotoxicological responses, since each tissue is structurally and functionally distinct and, therefore, can respond differently to the presence of environmental stressors. As evidenced by our data, the differential tissue responses to stressors can translate into either an enhancement of biomarker levels (e.g. increase in CAT activity, LPO, Ub and VTG liver contents due to VFX exposure) or an inhibition (e.g. decreased CAT and SOD activities in muscle and gills, respectively, due to VFX exposure, warming or acidification). In addition, when multiple stressors interact with each other, such effects can be either exacerbated (e.g. CAT activity in fish muscle further decreased by the combination of VFX with acidification or warming) or attenuated/counteracted (GST activity in the liver was inhibited by acidification or warming, but such inhibition was attenuated by VFX feed exposure).

By integrating all tissue biomarker responses, it became evident that the physiological stress induced by VFX water exposure was more severe (i.e. higher mean IBR index value) compared to VFX feed exposure, regardless of seawater temperature and pCO_2 levels. As for the interactive effects of abiotic stressors, while warming was generally associated to a poorer fish physiological state, the negative impact of acidification was only clearly evident in fish gills. Finally, the combination of the three stressors corresponded to the most severe stress scenarios (particularly, following VFX water exposure), overall yielding higher IBR index values than treatments simulating stressors acting alone or the interaction of two stressors. Hence, the present results emphasize the importance of conducting multi-stressor ecotoxicological assessments to enable a deeper understanding of the consequences of climate change, as well as to develop region-specific mitigation strategies, since environmental stressors will rarely occur in isolation and their ecological impacts will not be felt in the same way across the planet.

Appendix A

See Appendix Tables A1–A4.

Acknowledgments

The research leading to these results has received funding from the European Union Seventh Framework Programme (FP7/2007–2013) under the ECsafeSEAFOOD project (grant agreement no. 311820). The present study was also funded by The Portuguese Foundation for Science and Technology (FCT), through the: i) strategic project UID/MAR/04292/2013 granted to MARE, ii) FCT/MCTES project UID/Multi/04378/2013 co-financed by the ERDF under the PT2020 Partnership Agreement POCI-01–0145-FEDER-007728 granted to the Applied Molecular Biosciences Unit- UCIBIO; iii) the contracts of AM (IF/00157/2014) and RR (IF/01373/2013) in the framework of the IF program; and iv) the PhD Grant of ALM (SFRH/BD/103569/2014) and the Post-PhD Grant of PA (SFRH/BPD/100728/2014). Finally, the authors would like to acknowledge Sparos Lda company for providing the experimental feeds, as well as the staff of IPMA-EPPO aquaculture pilot station and MARE-LMG for the technical support.

Ethical statement

Fish trials were carried out following the EU ethical requirements (EU Directive 2010/63), being approved by the Ethical Committee of the Faculty of Sciences of the Lisbon University, overseen by the Portuguese National Competent Authority (Direção-Geral de Alimentação e Veterinária, DGAV). All researchers involved in fish trials were previously accredited by the Federation of European Laboratory Animal Science Associations (FELASA).

Funding sources

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Table A1
Seawater physical and chemical parameters (mean ± standard deviation) in each treatment. Abbreviations: CTR – reference temperature and pH conditions (i.e. T = 19 °C and pH = 8.0 units); VFX-feed – fish exposure VFX via feed; VFX-water –VFX exposure via water; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 °C); TA – total alkalinity; P CO_2 - partial CO_2 pressure; T CO_2 - Total CO_2 concentration; HCO_3^- - bicarbonate; CO_3^{2-} - carbonate ion concentrations; ΩCal - calcite saturation state; ΩAra – aragonite saturation state.

	Control	VFX-water	VFX-feed	Acid	Acid + VFX-feed	Warm	Warm + VFX-feed	Acid + Warm	Acid + Warm + VFX-feed	Acid + Warm + VFX-water
Temperature (°C)	19.0 ± 0.2	19.0 ± 0.3	19.0 ± 0.1	19.0 ± 0.1	19.0 ± 0.1	24.0 ± 0.3	24.0 ± 0.2	24.0 ± 0.2	24.0 ± 0.2	24.0 ± 0.2
Salinity	35.2 ± 0.5	35.1 ± 0.7	35.1 ± 0.5	35.3 ± 0.6	35.1 ± 0.7	35.4 ± 0.8	35.5 ± 0.6	35.4 ± 0.8	35.3 ± 0.6	35.4 ± 0.8
pH	8.02 ± 0.01	8.05 ± 0.03	8.01 ± 0.04	7.61 ± 0.03	7.63 ± 0.02	8.01 ± 0.02	8.04 ± 0.03	7.62 ± 0.03	7.62 ± 0.04	7.62 ± 0.03
TA (μmol kg^{-1})	2737.1 ± 20.9	2705.9 ± 33.2	2715.4 ± 33.1	2687.5 ± 60.1	2648.0 ± 135.8	2624.0 ± 43.8	2681.2 ± 68.2	2565.5 ± 191.6	2494.5 ± 71.4	2565.5 ± 191.6
***P* CO_2** (μatm)	540.8 ± 4.6	534.6 ± 8.5	535.5 ± 6.8	1497 ± 32.9	1477.7 ± 78.4	509.5 ± 9.4	523.2 ± 12.7	1433.9 ± 107.8	1394.8 ± 39.0	1433.9 ± 107.8
***T* CO_2** (μmol kg^{-1})	2467.9 ± 21.2	2437.4 ± 33.8	2444.9 ± 29.3	2610.9 ± 59.7	2572.1 ± 134.5	2313.8 ± 41.9	2371.5 ± 60.1	2461.4 ± 185.9	2392.9 ± 68.2	2461.4 ± 185.9
***HCO*$_3^-$** (μmol kg^{-1})	2242.7 ± 20.5	2214.0 ± 32.4	2220.1 ± 25.5	2470.8 ± 56.7	2434.0 ± 127.6	2066.7 ± 38.8	2121.9 ± 52.3	2316.5 ± 174.5	2252.4 ± 63.6	2316.5 ± 174.5
CO_3^{2-} (μmol kg^{-1})	207.3 ± 0.5	205.9 ± 1.0	207.3 ± 3.7	90.7 ± 1.7	89.4 ± 4.3	232.5 ± 2.8	234.4 ± 7.5	103.7 ± 8.4	100.4 ± 3.7	103.7 ± 8.4
Ω Ara	3.2 ± 0.1	3.2 ± 0.2	3.2 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	3.7 ± 0.0	3.7 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.1
Ω Cal	4.9 ± 0.0	4.9 ± 0.1	4.9 ± 0.1	2.2 ± 0.0	2.1 ± 0.1	5.6 ± 0.1	5.6 ± 0.2	2.5 ± 0.2	2.4 ± 0.1	2.5 ± 0.2

Table A2
Proximate chemical composition of CTR and VFX-enriched feeds (extracted from Maulvault et al., 2018b).

	%
Fishmeal LT70[a]	28.0
Fishmeal 60[b]	20.0
Fish protein concentrate[c]	2.5
Soy protein concentrate[d]	5.0
Wheat gluten[e]	5.5
Corn gluten[f]	5.0
Soybean meal 48[g]	9.0
Wheat meal[h]	5.0
Whole peas[i]	5.0
Fish oil[j]	13.5
Guar gum[k]	0.5
Vitamin and mineral premix[l]	1.0

[a] Peruvian fishmeal LT: 67% crude protein (CP), 9% crude fat (CF), EXALMAR, Peru.
[b] Fair Average Quality (FAQ) fishmeal: 62% CP, 12% CF, COFACO, Portugal.
[c] CPSP 90: 84% CP, 12% CF, Sopropêche, France.
[d] Soycomil P: 65% CP, 0.8% CF, ADM, The Netherlands.
[e] VITAL: 85.7% CP, 1.3% CF, ROQUETTE, France.
[f] GLUTALYS: 61% CP, 6% CF, ROQUETTE, France.
[g] Dehulled solvent extracted soybean meal: 47% CP, 2.6% CF, Cargill, Spain.
[h] Whole wheat: 10.2% CP, 1.2% CF, Casa Lanchinha, Portugal.
[i] Whole peas: 19.8% CP, 2.2% CF, Casa Lanchinha, Portugal.
[j] SAVINOR UTS, Portugal.
[k] Guar gum HV109, SEAH International, France.
[l] PREMIX Lda, Portugal: Vitamins: DL-alpha tocopherol acetate, 100 mg kg^{-1}; sodium menadione bisulphate, 25 mg kg^{-1}; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg kg^{-1}; riboflavin, 30 mg kg^{-1}; pyridoxine, 20 mg kg^{-1}; cyanocobalamin, 0.1 mg kg^{-1}; nicotinic acid, 200 mg kg^{-1}; folic acid, 15 mg kg^{-1}; ascorbic acid, 500 mg kg^{-1}; inositol, 500 mg kg^{-1}; biotin, 3 mg kg^{-1}; calcium panthotenate, 100 mg kg^{-1}; choline chloride, 1000 mg kg^{-1}, betaine, 500 mg kg^{-1}. Minerals: copper sulphate, 9 mg kg^{-1}; ferric sulphate, 6 mg kg^{-1}; potassium iodide, 0.5 mg kg^{-1}; manganese oxide, 9.6 mg kg^{-1}; sodium selenite, 0.01 mg kg^{-1}; zinc sulphate,7.5 mg kg^{-1}; sodium chloride, 400 mg kg^{-1}; excipient wheat middlings.

Table A3
Tissue molecular biomarker levels in *A. regius* from CTR treatment (average ± standard deviation; $n = 6$). Abbreviations: CAT – catalase; SOD – superoxide dismutase; GST – glutathione S-transferase; LPO - lipid peroxidation; HSP70/HSC70 – heat shock proteins; Ub – total ubiquitin; AChE – acetylcholinesterase; VTG – vitellogenin content.

	CAT activity (U mg^{-1} protein)	SOD activity (% inhibition)	GST activity (U mg^{-1} protein)	LPO (U mg^{-1} protein)	HSP70/HSC70 concentration (U mg^{-1} protein)	Ub concentration (U mg^{-1} protein)	VTG concentration (U mg^{-1} protein)	AChE activity (U mg^{-1} protein)
Muscle	14.3 ± 1.2	80.9 ± 2.3	26.1 ± 3.4	0.005 ± 0.001	1.7 ± 0.1	0.14 ± 0.02	–	–
Gills	13.3 ± 4.7	86.7 ± 2.4	13.7 ± 3.3	0.014 ± 0.005	1.1 ± 0.1	0.08 ± 0.03	–	–
Liver	21.8 ± 2.3	77.2 ± 7.6	26.4 ± 3.7	0.016 ± 0.002	1.8 ± 0.2	0.07 ± 0.04	25.9 ± 3.1	–
Brain	4.5 ± 1.3	67.7 ± 5.3	9.7 ± 0.6	0.002 ± 0.001	0.6 ± 0.2	0.03 ± 0.01	–	419.0 ± 43.4

Table A4
Molecular biomarker scores calculated in each treatment and fish tissue. Values in bold correspond to individual biomarker scores (S) that differed ≥ 0.5 from the CTR treatment score. Abbreviations: CAT – catalase activity; SOD – superoxide dismutase activity; GST – glutathione S-transferase activity; LPO - lipid peroxidation, measured as MDA concentration; HSP70/HSC70 - heat shock proteins concentration; Ub - total ubiquitin concentration; VTG – vitellogenin concentration; AChE – acetylcholinesterase activity; CTR – reference temperature and pH conditions (i.e. T = 19 °C and pH = 8.0 units); VFX-feed – VFX exposure via feed; VFX-water – VFX exposure via water; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 °C).

		CAT	SOD	GST	LPO	HSP70/HSC70	Ub	VTG	AChE
CTR	**Muscle**	2.79	2.34	0.62	0.52	1.00	1.31	–	–
	Gills	2.17	1.31	2.80	0.31	0.93	0.63	–	–
	Liver	0.49	1.56	2.02	1.71	1.15	0.91	1.42	–
	Brain	0.74	1.29	0.21	1.16	0.14	0.46	–	0.19
VFX-Feed	**Muscle**	**1.30**	**4.95**	**2.17**	**2.32**	**1.94**	**2.94**	–	–
	Gills	**3.81**	**4.67**	**2.24**	**2.98**	**1.56**	**1.71**	–	–
	Liver	**3.60**	**2.98**	2.30	**2.92**	1.48	**3.02**	**3.42**	–
	Brain	**2.02**	**0.52**	**1.85**	**3.35**	**1.71**	**2.26**	–	**2.16**
VFX-water	**Muscle**	**0.74**	**4.49**	**3.21**	**1.45**	**2.41**	**2.76**	–	–
	Gills	**2.69**	**4.45**	**2.08**	**2.39**	**3.09**	**2.47**	–	–
	Liver	**1.21**	**3.49**	**2.71**	**3.78**	**1.72**	**3.79**	**2.17**	–
	Brain	0.68	**2.72**	**2.33**	**3.13**	**3.09**	**4.28**	–	**2.57**
Acid	**Muscle**	**1.60**	**3.98**	0.20	**1.87**	0.65	**2.17**	–	–
	Gills	**1.32**	**4.14**	**4.67**	**2.28**	0.50	**3.49**	–	–
	Liver	0.56	**2.80**	**2.81**	**2.44**	**3.34**	**2.96**	**3.16**	–
	Brain	0.41	**2.62**	**2.31**	**4.05**	**1.95**	**2.05**	–	**2.12**
Acid + VFX-feed	**Muscle**	**0.79**	**4.53**	**2.14**	**3.20**	**1.92**	**2.77**	–	–
	Gills	**1.06**	**4.08**	**1.89**	**2.23**	**1.59**	**1.55**	–	–
	Liver	**1.16**	**4.35**	2.04	**3.85**	**1.80**	**3.52**	**2.10**	–
	Brain	**1.78**	**2.80**	**1.96**	**3.24**	**1.98**	**2.03**	–	**1.58**
Warm	**Muscle**	**1.61**	**5.22**	**2.18**	0.29	**2.60**	**3.71**	–	–
	Gills	**2.28**	**4.26**	2.31	**2.32**	0.78	**1.60**	–	–
	Liver	**1.34**	**2.81**	1.77	**3.93**	**2.42**	**2.32**	**2.18**	–
	Brain	**2.38**	**3.38**	**3.39**	**4.33**	**2.26**	**1.93**	–	**2.80**
Warm + VFX-feed	**Muscle**	**1.18**	**3.64**	**2.54**	**1.80**	**3.25**	**2.19**	–	–
	Gills	**2.73**	**4.91**	**0.61**	**2.39**	**1.49**	0.30	–	–
	Liver	**1.55**	**3.51**	**2.92**	**2.70**	**2.55**	**3.00**	**3.41**	–
	Brain	**2.29**	**2.15**	**1.64**	**2.86**	**1.82**	**1.77**	–	**1.56**
Acid + Warm	**Muscle**	**1.02**	**4.82**	**2.19**	**1.58**	0.76	**2.37**	–	–
	Gills	**1.97**	**4.39**	2.37	**2.49**	**0.24**	**1.55**	–	–
	Liver	**1.13**	**3.56**	**2.63**	**2.49**	**2.26**	**2.45**	**1.95**	–
	Brain	**1.89**	**3.00**	**1.42**	**2.83**	**2.57**	**2.07**	–	**2.87**
Acid + Warm + VFX-feed	**Muscle**	**3.49**	**3.77**	**2.72**	**2.92**	0.69	**2.62**	–	–
	Gills	**2.86**	**4.08**	2.34	**3.49**	**1.47**	**0.38**	–	–
	Liver	**1.72**	**3.78**	1.99	**3.03**	**2.89**	**2.72**	1.85	–
	Brain	**2.14**	**2.64**	**1.56**	**2.91**	**3.46**	**1.99**	–	**1.69**
Acid + Warm + VFX-water	**Muscle**	**0.25**	**4.02**	**3.28**	**2.08**	**3.06**	**3.90**	–	–
	Gills	**3.95**	**4.29**	2.35	**2.69**	**3.13**	**1.95**	–	–
	Liver	**1.64**	**4.06**	**2.73**	**2.93**	**2.17**	**2.95**	**3.10**	–
	Brain	**3.78**	**3.40**	**2.05**	**4.01**	**2.06**	**2.31**	–	**2.66**

Appendix B. Methodologies

Total protein content

Bradford assay (Bradford, 1976) was carried out to quantify total protein levels in each sample, and to enable the subsequent normalization of biomarker (i.e. given in mg of protein). In this assay, 180 µL of Bradford reagent (Bradford, 1976) and 20 µL of sample/standard were added to each microplate well. A calibration curve was generated with bovine serum albumin (BSA; Sigma Aldrich, Germany) at different dilutions (at least 7 concentrations, ranging from 0 to 2.0 mg mL^{-1}) as standard. Absorbance was read at 595 nm in a microplate reader (BioRad, Benchmark, USA).

Catalase (CAT)

Catalase activity (EC 1.11.1.6) was carried out following the procedure described by Johansson and Borg (1988), and adapted to 96-well microplates. To each microplate well, 20 µL of sample/standard, 100 µL of assay buffer (100 mM potassium phosphate; Sigma Aldrich, Germany), 30 µL of methanol (Merck, USA) and 20 µL of hydrogen peroxide (0.035 M; Sigma-Aldrich, Germany) were added. After 20 min incubation, 30 µL of potassium hydroxide (10 M; Merck, USA) and 30 µL of Purpald (34.2 mM; Merck, USA) were added. After incubating 10 min, 10 µL of potassium periodate (65.2 mM; Merck, USA) were added to finalize the reaction. A calibration curve was built using formaldehyde standards, with concentrations ranging from 5 to 75 µM of formaldehyde (Sigma Aldrich, Germany). Standard bovine catalase solution of 1523.6 U mL^{-1} (Sigma Aldrich, Germany) was used as positive control. Enzyme activity was calculated considering that one unit of catalase is defined as the amount that will cause the formation of 1.0 nmol of formaldehyde per minute at 25 °C. Absorbance was read at 540 nm and results expressed as µM min^{-1} mg $protein^{-1}$.

Superoxide dismutase (SOD)

Superoxide dismutase activity (EC 1.15.1.1) was carried out as described by Sun et al. (1988), and adapted to 96-well microplates. Briefly, 10 µL of sample were transferred to the microplate wells. In each well, 240 µL of a reagent mix (3 mM EDTA, Merck, USA; 3 mM xanthine, Sigma Aldrich, Germany; 0.75 mM NBT, Merck, USA; and 100 µm XOD Sigma Aldrich, Germany) were added. Negative controls (i.e. mix without sample) were also included. Samples absorbance was read at 550 nm, and results were presented as the percentage of enzyme inhibition, using the following equation:

$$\%\ \ inhibition = \frac{Abs550/min_{negative\ control} - Abs550/min_{sample}}{Abs550/min_{negative\ control}} x\ 100$$

Glutathione S-transferase (GST)

Glutathione S-transferase activity (EC 2.5.1.18) was determined according to the method described by Habig et al. (1974) and adapted to 96-well microplates. To each microplate well, 20 µL of sample/standard and 180 µL of a reagent mix (200 mM reduced L-glutathione, 100 mM CNDB and buffer Dulbecco; all from Sigma-Aldrich, Germany) were added. Equine liver GST (Sigma-Aldrich, Germany) was used as standard and positive control. Absorbance was read at 340 nm every minute during 6 min, with the increase in absorbance being directly proportional to GST activity. The reaction rate was determined considering the molar CDNB extinction coefficient of $0.0053\varepsilon^{mM}$, and results were expressed as nmol min^{-1} mg $protein^{-1}$.

Lipid peroxidation (LPO)

Lipid peroxidation was determined using a 96-well microplates protocol adapted from the thiobarbituric acid reactive substances (TBARS) method of Uchiyama and Mihara (1978). 5 µL of each sample/standard were added to microtubes. Then, 45 µL of monobasic sodium phosphate buffer (50 mM, Sigma Aldrich, Germany), 12.5 µL of SDS (8.1%, Sigma Aldrich, Germany), 93.5 µL of trichloroacetic acid (20%, pH1/43.5, Merck, USA), 93.5 µL of thiobarbituric acid (1%, Merck USA) and 50.5 µL of Milli-Q grade ultrapure water were added to each microtube. Microtubes were then vortexed for 30 s and incubated in boiling water for 10 min (microtubes' lids were punctured with a needle before incubation). Afterwards, microtubes were immediately placed on ice for a few minutes to cool to room temperature, and 62.5 µL of Milli-Q grade ultrapure water and 312.5 µL of n-butanol pyridine (15:1, v/v, MercK, USA) were added. Microtubes were vortexed again, and centrifuged at 10,000*g* for 5 min. Duplicates of 150 µL of the supernatant of each reaction were transferred to microplate wells. Lipid peroxides were quantified using an eight-point calibration curve ranging from 0 to 0.3 mM TBARS, performed with malondialdehyde bis (dimethylacetal) standards (Merck). Absorbance was read at 530 nm and results were expressed as nmol mg^{-1} protein.

Heat shock response (HSP70/HSC70)

Heat Shock Protein 70 (HSP70/HSC70) was quantified using an indirect Enzyme Linked Immunosorbent Assay (ELISA) based on a protocol from Njemini et al. (2005). Briefly, 50 µL of sample/standard were added to microplate wells, and left to incubate overnight at 4 °C. Afterwards, microplates were washed 3 times with a PBS solution containing 0.05% of Tween-20 (Sigma-Aldrich, Germany), and then blocked by adding 200 µL of 1% BSA solution prepared in PBS. Microplates were incubated for 90 min at 37 °C and washed again (with PBS + 0.05% of Tween-20 solution) and 50 µL of primary antibody solution were added to each well (anti-Hsp70/Hsc70, Acris, USA; diluted to 1.0 µg mL^{-1} in a 1% BSA solution). Then, microplates were incubated again for 90 min at 37 °C, washed (with PBS + 0.05% of Tween-20 solution) and 50 µL of secondary antibody solution were added to each well (anti-mouse IgG, fab specific, alkaline phosphatase conjugate, Sigma-Aldrich, Germany; also diluted to 1.0 µg mL^{-1} in 1% BSA solution). After incubating at 37 °C for 90 min, microplates were washed again, 100 µL of substrate (SIGMA FAST™ p-Nitrophenyl Phosphate Tablets, Sigma- Aldrich, USA) was added and microplates were left to incubate for 30 min at room temperature. Then, 50 µL of NaOH solution (3 N, Merck, Germany) were added to stop the reaction.

A calibration curve was performed using serial dilutions (at least 7 different concentrations) of purified HSP70 active protein (Acris, USA), ranging from 0 to 2.0 µg mL^{-1} of protein. The absorbance was read at 405 nm and results were expressed in µg mg^{-1} protein.

Ubiquitin (Ub)

Total ubiquitin was quantified using a direct ELISA method previously described by Madeira et al. (2014). Briefly, 50 µL of sample/standard were added to microplate wells, and left to incubate overnight at 4 °C. Afterwards, microplates were washed 3 times with a PBS solution containing 0.05% of Tween-20, and then blocked by adding 200 µL of 1% BSA solution prepared in PBS. Microplates were incubated for 90 min at 37 °C and washed again (with PBS + 0.05% of Tween-20 solution) and 50 µL of a conjugated primary antibody (Ub P4D1, sc-8017, HRP conjugate, Santa Cruz, USA) were added to each well. Microplates were incubated for 90 min at 37 °C, washed again (with PBS + 0.05% of Tween-20 solution), and 100 µL of substrate (TMB/E, Temecula California, Merck Millipore) was added. After incubating for 30 min at room temperature, 100 µL stop solution (1 N HCl, Merck, USA) was added. A calibration curve was performed using serial dilutions (at least 7 concentrations) of the ubiquitin standard E110, UbP (Santa Cruz, USA; concentrations ranging from 0 to 2.0 µg mL^{-1} protein). The absorbance was read at 450 nm and results were expressed in µg mg^{-1} protein.

Acetylcholinesterase (AChE) activity

Acetylcholinesterase (EC 3.1.1.7) activity was assessed in brain tissues, following a methodology adapted from Ellman et al. (1961) to 96-well microplates. Briefly, 50 µL of previously diluted samples (1:10) were transferred to the microplate wells, and 250 µL of a reagent mix containing (sodium phosphate buffer 50 mM at 8.0 pH units, 1 mM Ellman's reagent, i.e. DTNB – 5,5′-dithiobis-(2-nitrobenzoic acid; in 50 mM phosphate buffer, and 75 mM ACTI (acetilthiocholine iodide); in phosphate buffer 50 mM; all from Sigma-Aldrich, Germany) were added. Negative controls were

included (i.e. 50 µL of buffer instead of sample). Samples absorbance was read at 412 nm, every minute during 10 min, and AChE activity was measured considering that one unit of enzyme is responsible for the formation of 1.0 µmol of thiocholine per minute. Results were expressed as nmol min^{-1} mg $protein^{-1}$.

Vitellogenin (VTG)

Vitellogenin content (VTG) was determined in fish liver following a direct ELISA assay based on the protocol of Denslow et al. (1999). Briefly, 50 µL of sample (previously diluted 1:200) or standard were added to microplate wells, and left to incubate overnight at 4 °C. Afterwards, microplates were washed 3 times with a TBST solution (10 mM Tris-HCl at 7.0 pH units, Merck, USA; 150 mM NaCl, Merck, USA; and 0,05% de Tween-20, Sigma-Aldrich, Germany), and then blocked by adding 200 µL of 1% BSA solution prepared in PBS. Microplates were incubated for 90 min at 37 °C and washed again (with TBST solution) and 50 µL of a primary antibody (carp VTG monoclonal antibody, Biosense, Norway; diluted to 1.0 µg mL^{-1} in a 1% BSA solution) were added. Then, microplates were incubated again for 90 min at 37 °C, washed (TBST solution) and 50 µL of secondary antibody solution (anti-mouse IgG, fab specific, alkaline phosphatase conjugate, Sigma-Aldrich, Germany; also diluted to 1.0 µg mL^{-1} in 1% BSA) were added to each well. After incubating at 37 °C for 90 min, microplates were washed again, 100 µL of substrate (SIGMA FAST™ p-Nitrophenyl Phosphate Tablets, Sigma- Aldrich, USA) was added and microplates were left to incubate for 30 min at room temperature. Then, 50 µL of NaOH solution (3 N) were added to stop the reaction. A calibration curve was performed using serial dilutions (at least 7 concentrations) of carp VTG standard (Biosense, Norway), ranging from 0 to 2.0 µg mL^{-1} of protein. The absorbance was read at 405 nm and results were expressed in µg mg^{-1} protein.

Integrated Biomarker Response (IBR)

Integrated biomarker responses (IBRs) and respective star plots were calculated in order to understand the global ecotoxicological responses of *A. regius* exposed to VFX (via feed and water), acidification and warming. Calculations were performed according to the methodology proposed by Beliaeff and Burgeot (2002), later modified by Guerlet et al. (2010). Briefly, for each tissue, the general mean (m) and standard deviation (s) of a given biomarker was calculated (including data from all treatments), and subsequently standardized to obtain Y, i.e. $Y = (X - m)/s$, where X is the mean biomarker value of a given treatment. Then, Z was calculated as Z = -Y or Z = +Y according to the expected biological effect, with "-" representing an inhibition of a biological effect and "+" representing an induction (such evaluation was based on the average baseline biomarker values). Then, biomarker scores (S) were calculated as $S = Z + |Min|$, where $Z \geq 0$ and $|Min|$ is the absolute value of all Y calculated for a given biomarker (including all measurements). Star plots were performed to represent the scores (S) of all biomarkers measured in a given treatment and tissue, as well as to calculate IBRs according to the following formulas:

$$IBR = \sum_{t=1}^{n} A_i A_i = \frac{1}{2}\sin\left(\frac{2}{n}x\pi\right) x S_i x S_{i+1}$$

where, A_i is the area connecting the two scores (S), S_i and S_{i+1} are two consecutive clockwise scores (radius coordinates) of a given star plot, and n is the number of biomarkers used for calculations. IBR calculations were always performed with the same order of parameters for all treatments and tissues, i.e. CAT, SOD, GST, LPO, HSP70/HSC70, Ub, and VTG (in the case of fish liver) or AChE (in the case of fish brain). In order to evaluate the scores as a fitness index, values that differed in 0.5 from the score of CTR treatment were considered to be from an animal with a higher or lower fitness (Ferreira et al., 2015).

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